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The Chemical and Physical Characteristics of Preparations Containing the Milk Agent Virus: *A Review**

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Although the purpose of this review is to examine the available data concerning the chemical nature of the virus involved in mammary carcinogenesis in mice, a short review of some of the more general aspects of the problem of mouse mammary cancer appears to be a necessary starting point. The spontaneous development of mammary carcinoma in a high percentage of any stock of mice is dependent upon three major factors: a genetic susceptibility to the development of breast cancer, a proper hormonal constitution, and the milk agent virus which is passed from mother to offspring during nursing (10, 14). The absence of any one of these factors to a great degree precludes the development of a high incidence of breast cancer, and for this reason it would seem impossible to designate one or another as the inciting cause of mammary cancer. Other factors such as nutrition (36, 38, 42), temperature (21, 37, 41), and animal crowding (2) can affect the final incidence of cancer to greater or lesser degrees, but there is evidence to suggest that their action may be mediated through one of the three major factors, often via the endocrine system (25, 30, 43).

In the years since Bittner first demonstrated that the extra-chromosomal influence (27, 31) was milk-borne (9), evidence for the viral nature of this milk agent has accumulated. It was early demonstrated that the infective principle would pass through a bacterial (Berkefeld or Seitz) filter (3, 13) and, somewhat later, that infectivity of tissue extracts was concentrated in the pellets after centrifugation in gravitational fields ranging from 15,000 to 110,000 *g* (19, 39). These observations established the size of the infective particle to be within the size range of known filtrable viruses. Other work indicated that infectivity was retained after short periods of storage in glycerin (3, 13) and after freezing and lyophilization (11) but was lost upon heating at 56–60° C. for 30–60 minutes (3, 5). The agent was shown to be antigenic when introduced into animals other than the mouse, and it was demonstrated that antibodies so produced could neutralize the infectivity of the agent *in vitro* (3, 24). It was also found to be stable when exposed to a wide range of hydrogen ion concentrations, retaining infectivity in solutions ranging from pH 5 to 10.2 (5). These findings seem to establish the viral nature of the milk agent. For more complete considerations of the biological characteristics of the milk agent, the reader is referred to recent reviews by Andervont (1) and Bittner (16, 17).

In any consideration of the chemical composition of a virus, due concern must be paid to the purity of the preparation analyzed. Certain established criteria of purity of animal viruses have been reviewed by Beard (8). Physical homogeneity as judged by ultracentrifugation and/or electrophoresis is a cardinal requirement when purity is being considered. Recently, use of the electron microscope has added the criterion of morphological homogeneity. Beyond these physical criteria, Beard states that "virus is recognizable unequivocally only by its capacity to cause disease, and for this reason any biological criterion must be based ultimately on infectiousness." Owing to the pro-

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longed latent period required for the development of breast cancer in mice, the assay of biological activity of preparations suspected of representing a concentrated or purified milk agent virus is very difficult, although its importance remains paramount.

Concentration of viral activity by centrifugal technics.—It has been demonstrated by several investigators that the milk agent virus can be sedimented in relatively high centrifugal fields. Visscher *et al.* (39, 40) found virus activity in pellets obtained by spinning extracts of mammary gland tissue at 15,000 *g* for 30 minutes and at 110,000 *g* for 1 hour with but little activity in the final supernatant fraction. Working with mouse milk, Bryan *et al.* (19) demonstrated virus activity in a pellet obtained at 60,000 *g* for 1 hour but also found some activity in the supernatant fluid. In both these investigations, relatively large doses of the various fractions were injected.

Kahler and Andervont (28) subjected milk from C3H mice to a centrifugal field of 28,000 *g* for 40 minutes and interpreted their infectivity data as suggesting that the sedimenting boundary of virus had passed well below the lower one-fifth of the fluid in the tube. As reported earlier (4), by using technics of differential centrifugation combined with titration experiments involving serial dilution of the isolated fractions, it was proposed that the supernatant fluid, obtained after spinning saline extracts of spontaneous mammary carcinoma tissue at 23,000 *g* for 1 hour, was essentially free of virus activity, while the sediments contained appreciable activity. This has been more clearly demonstrated in this laboratory in an experiment in which the lactating mammary glands of two AZF_1 mice were used as the starting material. The young were allowed to suckle up to the time of sacrifice, so the glands contained little milk. The gland tissue was ground in a mortar, with saline buffered at pH 7, and cleared at 23,000 *g* for 5 minutes. This supernatant solution was then spun at 23,000 *g* for 90 minutes to sediment the microsome pellets which were resuspended and resedimented at 23,000 *g* for 90 minutes. The microsomal supernate, which had been carefully withdrawn through a syringe and fine gauge needle from beneath the fatty surface layer, was also respun 90 minutes at 23,000 *g* and again carefully withdrawn from the center portion of the tube. These two fractions, the microsomes and the microsomal supernate, were diluted in buffer at pH 7 and injected intraperitoneally into test mice. The results are recorded in Table 1. As seen in Table 1, if we take 5×10^{-5} gm. equivalents to be the endpoint in the titration of the microsome fraction, the supernatant fraction contains less than 0.25 per cent of the virus activity of the microsome fraction.

It would seem, then, that the milk agent virus can essentially be completely sedimented in a centrifugal field of 23,000 *g* during a period of 90 minutes. In the experiments cited (19, 40), in which tumors developed in mice injected with supernatant fluids obtained after sedimenting runs of 60,000 and 110,000 *g* for 1 hour, the amounts of material injected were derived from 0.2 cc. of milk and 1 gm. of breast tissue, respectively. Since titration experiments (26) have shown that milk and mammary gland extracts can give rise to tumors in dilutions representing 10^{-6} gm., it is clear that the ultracentrifugal supernates from 0.2 cc. of milk or 1 gm. of breast tissue extract might contain only 0.0005–0.0001 per cent of the total virus activity and still be expected to cause tumors. Contamination of the supernatant fluid of this magnitude could easily result from virus particles that had been carried to the top of the tube in association with fat droplets or had been resuspended from the pellet during deceleration or decantation.

TABLE 1

TITRATION OF MILK AGENT VIRUS ACTIVITY IN MICROSOMES AND MICROSOMAL SUPERNATE DERIVED FROM LACTATING MAMMARY-GLAND TISSUE OF AZF_1 MICE

Fraction	N (Mg/gm of wet tissue)	Pentose (Mg/gm of wet tissue)	Gram equiv. injected	Mice living to 1st tumor	Per cent with tumors
Microsomes	3.24	1.49	5×10^{-3}	26	89
			5×10^{-4}	35	74
			5×10^{-5}	31	68
			5×10^{-6}	34	35
Supernate			2×10^{-2}	9	0

Chemical composition of concentrates containing the milk agent virus.—The milk agent virus can be sedimented from saline extracts at 23,000 *g* for 90 minutes along with the microsome fraction of the tissue, and, therefore, it becomes of interest to compare the chemical composition of such microsome fractions[1] from mammary glands with and without the agent. In Table 2 are recorded some chemical data on microsomes isolated from essentially milk-free lactating mammary glands of A and Z mice compared with similar fractions from Ax and Zb mice.[2] It will be noted that the absolute amounts (mg. per gram tissue) of the various

[1] Kahler and Bryan (29) have also studied the sedimenting characteristics of macromolecular particles in mouse milk and mammary tumor extracts and demonstrated the presence of nucleic acid in centrifugally prepared pellets.

[2] Ax and Zb mice are genetically identical to A and Z mice, respectively, but lack the milk agent (12, 15).

constituents show considerable variation from experiment to experiment. This is probably due to the difficulty of complete homogenization of mammary gland tissue. However, the percentage composition and the ratios of one constituent to another show much less variation, and the averages for glands with and without the agent show no significant differences.

These microsome fractions from lactating mammary glands differ somewhat from similar fractions isolated from mouse liver tissue (6). The principal difference is in the pentose nucleic acid (PNA) content, which for liver microsomes is 9.1 per cent, as against 14.9 per cent for mammary gland microsomes. It has been noticed that liver microsomes can be resuspended twice in either neutral saline or distilled water and resedimented each time at 23,000 *g* for 90 minutes with but small loss of total substance and with no significant change in composition. The microsome fraction from lactating mammary tissue, on the other hand, is much more erratic in its behavior and frequently appears quite unstable. In one instance, two washes with a buffer at pH 7 removed 72 per cent of the PNA and 59 per cent of the protein originally present in the fraction.

While neutral solutions frequently extract PNA from mammary gland microsomes, it is invariably possible to almost completely extract the PNA by the use of alkaline wash solutions. By washing microsomes obtained from mammary glands, with and without the milk agent virus, in a carbonate-bicarbonate buffer at pH 10 (0.1 M), we have obtained a fraction still sedimentable at 23,000 *g* for 90 minutes which appears to be essentially a lipoprotein complex almost devoid of PNA. Analyses of these fractions were carried out by the Schneider procedure (35) and are recorded in Table 3. Again, no significant differences have as yet been observed between such fractions obtained from glands with and without the milk agent virus.

Since this washing at pH 10 did such an efficient job of separating most of the PNA and some of the protein from the bulk of the phospholipide and the remaining protein, it was of interest to determine the fate of the virus activity: that is, was it destroyed or, if not, was it associated with the "nucleoprotein" or the "lipoprotein" fraction of the microsomes. Table 4 records the results of a titration experiment in which microsomes were isolated from the lactating glands of two AZF_1 mice and were then washed, via centrifugation, at 23,000 *g* for 90 minutes, with buffers at pH 7 or pH 10.

As may be seen in Table 4, the dilutions were not carried far enough to reach a 50 per cent end-point, but it is clear that the pH 10 extract, which contains the bulk of the PNA, is essentially devoid of virus activity. Furthermore, there is no indication that the microsomal sediment, after extraction at pH 10, has any less virus activity than the original microsome fraction.

In this experiment, in which the end-point unfortunately was not reached, tumors developed in fifteen out of eighteen animals that received from 7 to 12 mμg. of nitrogen. In other experiments (26), six out of fourteen animals receiving 2.3 mμg. of nitrogen, and four out of fourteen animals receiving 0.23 mμg. of nitrogen developed tumors. Since no significant chemical or electron microscopic (26) differences have yet been observed between fractions showing such virus activity and comparable fractions obtained from mice that do not possess the milk agent, we are led to believe that these fractions, even though they show virus activity in high dilution, are grossly impure concentrates.

TABLE 2

COMPOSITION OF MICROSOME FRACTIONS ISOLATED FROM LACTATING MAMMARY GLANDS OF MICE WITH AND WITHOUT THE MILK AGENT VIRUS

Mice used	N (Mg/gm wet tissue)	P (Mg/gm wet tissue)	Pentose (Mg/gm wet tissue)	Pent. P as per cent tot. P	N/P	N/pent	N (Composition of fraction based on its dry weight)	P (Composition of fraction based on its dry weight) (per cent)	PNA (Composition of fraction based on its dry weight) (per cent)
A and Z	*2.35* (5)*	*0.49* (5)	*1.48* (5)	*62.4* (5)	*4.87* (5)	*1.61* (5)	*11.2* (2)	*2.4* (2)	*14.9* (2)
	1.8–2.8	0.33–0.61	1.0–1.9	57–66	4.2–5.5	1.4–1.8	11.1–11.4	2.0–2.7	13.5–16.3
Ax and Zb	*2.69* (5)	*0.57* (5)	*1.63* (5)	*58.6* (5)	*4.71* (5)	*1.66* (5)	*11.4* (2)	*2.6* (2)	*14.9* (2)
	1.3–3.6	0.30–0.75	0.7–2.1	52–65	4.3–5.2	1.6–1.7	11.0–11.8	2.5–2.7	13.7–16.0

* Figures are averages of the number of experiments shown in parentheses. Ranges are shown below each average.

TABLE 3

THE COMPOSITION OF PH 10 EXTRACTED MICROSOME FRACTIONS OBTAINED FROM LACTATING MAMMARY GLANDS

Prot. N (Mg/gm wet tissue)	Lipide P (Mg/gm wet tissue)	Pentose (Mg/gm wet tissue)
0.33 (5)	*0.10* (6)	*0.024* (9)
0.12–0.45	0.07–0.13	0.015–0.047

Graff *et al.* (22, 23), working with milk from Paris R III mice, have isolated, after treatment with chymotrypsin, a particulate fraction sedimenting between 700 *g* for 10 minutes and 120,000 *g* for 30 minutes. They have subjected this particulate fraction to electrophoretic and ultracentrifugal analyses and have observed it in the electron microscope. Electrophoretically, the fraction migrated as two components, and, similarly, in the ultracentrifuge, it sedimented as two components. In the electron microscope they observed irregular spheres with a size range from 50 to 150 mμ in diameter and an average size (calculated from their bar diagram) of 98.5 mμ. In milk from carcinoma-free C57 mice, subjected to the same treatment, they were unable to detect any such heavy particle fraction. However, from the milk of C57 mice that had been foster-nursed by Paris R III mice they were able to isolate a particulate fraction. This fraction showed electrophoretic and ultracentrifugal patterns comparable to the fraction from Paris R III milk, but the average diameter of the particles observed in the electron microscope was only 72.7 mμ.

In testing the biological activity, Graff *et al.* report that the injection of fractions containing 1–30 μg. of nitrogen and others containing 10 μg. of nitrogen have given rise to tumors in 49.4 and 46.4 per cent, respectively, of the test animals. They mention that carcinomas have been obtained after the injection of as little as 8 mμg. of nitrogen and that further dilution experiments are under way. Any evaluation of the purity of their fractions, compared to the presumably impure fractions reported in this paper, must await a demonstration that tumors in test animals can be produced by preparations containing very significantly less than 8 mμg. of nitrogen.

However, the demonstration, in the milk of infected R III mice, of a particle with the general physical characteristics of known viruses, and of its absence in the milk of uninfected C57 mice, is encouraging. The difference in particle size in preparations obtained from the milk of C57 mice infected with the R III virus is, on the other hand, somewhat confusing.

The size of the original R III particles is in rather good agreement with that of a morphologically characteristic particle observed by Porter and Thompson (34) in mammary carcinoma cells grown in tissue culture. This characteristic particle was seen in explants from three of six mammary carcinomas studied. The particles were bizonal in character, with an average outside diameter of 130 mμ and a denser central zone averaging 75 mμ in diameter. Several other viruses have been demonstrated to have a similar complexity of morphology (for review, see 8), which would seem to increase the probability that the particles observed by Porter and Thompson were viral in nature. The biological testing of these particles from tissue culture preparations appears, at the moment, to be difficult or impossible. The inability of these authors to find such particles in explants from three tumors might be interpreted (as we have interpreted our inability to identify characteristic particles in microsomal preparations of known biological potency) as indicating that the virus occurs very sparsely throughout the infected cells. In those explants in which Porter and Thompson observed characteristic particles, it seems possible that the normal cell-virus relationship may have been altered to favor viral multiplication. Thus, a considerable increase in the number of virus particles may have occurred in these explants, mak-

TABLE 4

ACTIVITY OF THE MILK AGENT VIRUS AFTER EXTRACTION AT PH 10*

	N (Mg/gm wet tissue)	Pentose	Gram equiv. injected	Mice living to 1st tumor	No. mice with tumors	Average tumor age (days)	Average age at death of nontumor mice (days)
Original microsomes	2.91	1.28	10^{-3}	17	17	262	
			10^{-4}	18	16	282	186
			10^{-5}	17	16	262	273
Microsomes washed twice at pH 7	1.17	0.36	10^{-3}	7	7	256	
			10^{-4}	9	9	308	
			10^{-5}	9	7	348	724
Microsomes washed at pH 10, then at pH 7	0.69	0.047	10^{-3}	8	8	268	
			10^{-4}	8	8	244	
			10^{-5}	9	8	294	684
Microsomal extract at pH 10	2.10	1.18	10^{-3}	9	1	222	621
			10^{-4}	9	0		704
			10^{-5}	9	0		650

* A preliminary report of these results was made at the Fourth International Cancer Research Congress, 1947 (7).

ing observation of the characteristic particles possible.

Recently, Passey *et al.* (32, 33) have reported observations in the electron microscope on extracts of tissues from several high- and low-cancer strains of mice. They prepared their extracts by grinding dried tissue with water (or with petroleum ether and then with water), clearing at 2,600 *g* for 30 minutes, and filtering through filter paper. In some experiments this filtrate was incubated with trypsin (30 minutes at 37° C.) and then filtered through a Berkefeld N candle. The final filtrates were examined under the electron microscope immediately and again after standing 4–14 days in an ice-chest.

They observed that the filtrates from lactating breast tissue and spontaneous tumor tissue of high-cancer strain mice contained particles, roughly spherical in shape, ranging in diameter from about 20 mμ to 120 mμ. The majority of particles were 30 mμ or less. Filtrates from lactating breast tissue of low-cancer strain mice contained few of these particles, especially after the filtrates had stood in the ice-chest. Preparations from high-cancer mice, after incubation with trypsin, gave much better electron micrographs, because the trypsin seemed to remove the tissue debris, making the particles more easily visible.

Extracts of spontaneous tumor tissue (presumably after incubation with trypsin) were centrifuged at 60,000 *g* for 2 hours, and the supernates still contained these particles. However, after 120,000 *g* for 2 hours the particles could not be demonstrated in the supernatant fluid. Biological tests, not yet complete, showed 56 of 221 test mice developing tumors when injected with extracts. There is no indication of the amount of material injected or whether these extracts had been subjected to centrifugation. Passey *et al.* make no claim that the particles they observed are the milk agent virus but are awaiting the final results of their biological tests.

Both Graff and Passey are dealing with particles which they feel are characteristic for tissues known to contain the milk agent virus. However, the particles of Graff range from 50 to 150 mμ in diameter and are destroyed by incubation with trypsin, while those of Passey are predominantly 30 mμ or less and are resistant to trypsin action. It seems unlikely that they are both observing the same particles, unless the differences in preparation, such as desiccation and incubation with enzymes, cause marked alterations in the physical characteristics.

From this brief review it may be concluded that the final judgment on the degree of purity of any preparation containing the milk agent virus must rest primarily on a determination of its specific infectivity, i.e., on its infectivity per unit weight (or per unit of nitrogen). Such specific infectivity can be determined, we believe, only by means of titration experiments involving injection of test mice with serial dilutions of the purified preparations. It is fully recognized that this procedure requires the use of large numbers of test mice and requires many months for its completion, but, unfortunately, no simpler yet conclusive procedures are available.

In studies with the virus of chicken tumor I, Bryan (18) found the latent period of tumor development an accurate index of the amount of virus injected. To see if this might also be the case with the milk agent virus as suggested in data presented by Bryan *et al.* (19), we analyzed data from five titration experiments carried out in this laboratory under as nearly standardized conditions as possible. The source material in all experiments was lactating mammae of A or AZF_1 females, and the test animals were ZBC females lacking the milk agent, injected with test solutions when 25–32 days of age, and then force bred for a 6-month period. In all experiments, 25–40 mice were injected with each dilution of virus, and in four of the experiments the 50 per cent end-point was reached or exceeded. Although the average age at which cancers developed did not increase with decreasing amounts of injected virus in all experiments, the average reciprocal of the latent period[3] decreased rather regularly with decreasing dosage. When the log of the dose was plotted against the reciprocal of the latent period, however, the slope of the regression line was not constant from experiment to experiment, nor was the reciprocal of the latent period for the 50 per cent end-point group the same in the different experiments. It would appear, then, that the latent period of mammary tumor development does not reflect the viral dosage accurately enough to be used in estimating the viral content of different preparations to be assayed.

The observations of particles of varying sizes, which appear to be characteristic of tissues and milk from mice infected with the milk agent virus, are encouraging beginnings in the purification of this virus. Even if certain of these particles, when

[3] The average reciprocal latent period was calculated as follows:

$$\frac{\Sigma\ 1{,}000/\text{lat. period in days}}{\text{corrected number of animals in the group}}.$$

The correction factor developed by Bryan and Shimkin (20) was used to properly weight age at death of the nontumorous animals.

ultimately isolated as homogeneous preparations, should fail to show a high specific infectivity, valuable information concerning the effect of the virus on the structural composition of the tissues will have been obtained. Perhaps the specific infectivity data, reported in this paper for impure concentrates of the milk agent virus, can serve as a useful base line for subsequent evaluations of the degree of purity of various preparations. At such time that it becomes possible to obtain a preparation with high specific infectivity, which also shows electrophoretic, ultracentrifugal, and morphological homogeneity, it will be feasible to consider the chemical composition of the virus.

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Macromolecular Particles Obtained from Human Neoplastic and Non-neoplastic Lymph Nodes

I. Procedure and Preliminary Results*

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INTRODUCTION

The present study concerns the segregation of dispersed macromolecular particles of all sizes, obtained from human neoplastic and non-neoplastic lymph node cells by means of a ten-step differential centrifugation procedure in which the suspending medium consists of a buffered, isotonic diluent containing an anti-coagulant. The preliminary results reported include the characterization of lymph node cell particles smaller than 350 mμ by electron microscopy and by a spectrophotometric and certain chemical procedures.

MATERIALS AND METHODS

The technics described in the present study involve the use of the following equipment and procedures: differential centrifugation using the Sorvall and International size I, type SB centrifuges for gravities of 1,500 and 3,000; the high speed attachment of the International size I, type SB centrifuge for gravities of 6,000, 11,000 and 25,000; and the spinning-top type air-driven ultra-centrifuge (3) for a gravity of 85,000. Visual examination of samples was made in the dark field attachment of the light microscope and the RCA type EMU electron microscope. Qualitative and quantitative nucleic acid determinations were carried out with the Beckman type DU spectrophotometer. The method of Schneider (13) was used to extract nucleic acid from centrifugation fractions in order to determine the presence of this substance qualitatively. The presence of ribose was determined by the orcinol (13) and phloroglucinol (8) reactions; desoxyribose by the diphenylamine (7) reaction; and purine bases by the copper sulfate (9) and ammoniacal silver nitrate (9) reactions.

Human lymph nodes obtained at surgery and necropsy (less than 2 hours after death) were stored at −65° C. until used. Tissue suspensions were prepared by grinding frozen lymph nodes (2.5 per cent to 10.0 per cent concentration) in a Waring Blendor for 3 minutes and were extracted for 18 hours prior to centrifugation. Tissue grinding, extraction, and centrifugation were conducted in a 4° C. cold room.

Centrifugation fractions were divided following preparation, so that concurrent electron microscope examination and spectrophotometric and chemical determinations could be made with the same lymph node preparation. A total of 46 neoplastic and 22 non-neoplastic lymph nodes were used in the present study.

EXPERIMENTAL PROCEDURES AND RESULTS

Preparation of tissue suspension.—The degree of agglutination of particles and tissue debris observed in samples of tissue suspensions prepared in 5 per cent concentration in fourteen different liquid media was recorded at intervals from 3 minutes to 96 hours with the dark field attachment of the light microscope (mag. ×950); the hydrogen ion concentration of tissues suspended in these media was also measured. All the diluents tested, with the exception of 0.1 M phosphate buffer, pH 8.0, and 0.1 M phosphate buffer, pH 8.0 containing heparin[1] (from 0.005 per cent to 0.3 per cent concentration), proved unsatisfactory as tissue suspending media, since agglutination of

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[1] The sodium heparin used in this experiment was supplied through the courtesy of the Upjohn Company, Kalamazoo, Mich.

TABLE 1

THE HYDROGEN ION CONCENTRATION AND DEGREE OF AGGLUTINATION OF CELLULAR PARTICLES OBSERVED IN UNCENTRIFUGED TISSUE SUSPENSIONS

	3 MINUTES		24 HOURS		48 HOURS		72–96 HOURS	
	pH*	Agg.	pH*	Agg.	pH*	Agg.	pH*	Agg.
0.1 M phosphate buffer, pH 8.0 containing 0.3 per cent heparin	8.0	0	7.9	0	7.8	0	7.7	0
0.1 M phosphate buffer, pH 8.0 containing 0.03 per cent heparin	7.9	0	7.9	0	7.8	0	7.7	0
0.1 M phosphate buffer, pH 8.0 containing 0.005 per cent heparin	8.0	0	7.9	0	7.7	0	7.7	1+
0.1 M phosphate buffer, pH 8.0	7.8	0	7.9	0	7.8	0	7.8	1+
0.05 M phosphate buffer, pH 8.0	7.8	0	7.6	0	7.5	1+	7.4	2+
0.1 M phosphate buffer, pH 7.0	7.0	0	7.0	0	7.0	0	6.9	1+
0.05 M phosphate buffer, pH 7.0	7.0	0	7.0	0	7.0	0	6.9	1+
0.005 M phosphate buffer, pH 8.0	7.5	0	7.3	0	7.0	0	6.9	3+
0.005 M phosphate buffer, pH 7.0	6.9	0	6.8	1+	6.8	1+	6.7	2+
0.6 M phosphate buffer, pH 7.0 containing 0.3 per cent heparin	6.9	0	6.8	0	6.6	2+	6.6	2+
0.85 per cent sodium chloride containing 0.3 per cent heparin	6.5	0	6.5	3+	6.3	3+	6.2	3+
0.85 per cent sodium chloride	6.8	0	6.5	2+	6.3	2+	6.2	4+
Ringer's solution	6.1	2+	6.6	3+	6.6	3+	6.5	4+
Ringer's solution containing 0.5 M ammonium hydroxide	7.4	1+	6.7	2+			6.7	3+

Agg. = agglutination.

0 = Absence of agglutination of cellular particles.

1+ to 4+ = Degree of agglutination of cellular particles. (Dark field examination; Mag. × 950.)

* = Beckman Glass Electrode pH Meter.

TABLE 2

CENTRIFUGATION PROCEDURES INVESTIGATED IN THE SEGREGATION AND VISUAL STUDY OF MACROMOLECULAR PARTICLES OBTAINED FROM HUMAN LYMPH NODE CELLS

	METHOD I	METHOD II		METHOD III		METHOD IV		METHOD V
I-Tissue Suspension A. Suspending Medium	Phosphate Buffer	Phosphate Buffer	Saline	Phosphate Buffer	Saline	Phosphate Buffer	Phosphate Buffer + 0.3% Heparin	Phosphate Buffer + 0.3% Heparin
1. Molarity or Per cent.	0.05 M	0.1 M	0.85%	0.1 M	0.85%	0.1 M	0.1 M	0.1 M
2. pH	7.0	8.0	6.5	8.0	6.5	8.0	8.0	8.0
B. Tissue Concentration-%.	10	5		5		5 ; 2.5		5
II- Differential Centrifugation Procedure	Tissue Suspension							
	1,500xg - 10 min. → S_1 / P_1	1,500xg - 10 min. → P_1 / S_1		3,000xg - 10 min. → P_1 / S_1		1,500xg - 10 min. → S_1 / P_1		1,500xg - 10 min. → S_1 / P_1
	85,000xg - 60 min. → P_2 / S_2	1,500xg - 10 min. → S_2 / P_2		3,000xg - 20 min. → S_2 / P_2		1,500xg - 30 min. → S_2 / P_2		1,500xg - 15 min. → S_2 / P_2
	1500xg - 10 min. → S_3 / P_3	25,000xg - 90 min. → P_3 / S_3		25,000xg - 90 min. → P_3 / S_3		1,500xg - 30 min. → S_3 / P_3		1,500xg - 20 min. → S_3 / P_3
	85,000xg - 60 min. → P_4 / S_4					3,000xg - 10 min. → S_4 / P_4		3,000xg - 10 min. → S_4 / P_4
						3,000xg - 30 min. → S_5 / P_5		3,000xg - 30 min. → S_5 / P_5
						3,000xg - 45 min. → S_6 / P_6		3,000xg - 45 min. → S_6 / P_6
						3,000xg - 60 min. → S_7 / P_7		6,000xg - 30 min. → S_7 / P_7
						6,000xg - 30 min. → S_8 / P_8		25,000xg - 90 min. → S_8 / P_8
						11,000xg - 30 min. → S_9 / P_9		1,500xg - 10 min. → S_9 / P_9
						25,000xg - 90 min. → P_{10} / S_{10}		25,000xg - 90 min. → P_{10} / S_{10}

Legend
S = Supernatant Fluid
P = Centrifugation Sediment
Min. = Minutes
○ Product used in the following step of the separation procedure.
□ Product discarded.

cellular particles and tissue debris occurred (Table 1).

Many of the tissue extracts prepared in 0.1 M phosphate buffer, pH 8.0, became gelatinous immediately following preparation, or on standing at 4° C. for a short period of time; because of the presence of a gel, it was impossible to sediment tissue debris and large particles at 1,500 *g*. When tissues were suspended in 0.1 M phosphate buffer, pH 8.0 containing heparin in a concentration of 0.03 per cent, gel formation failed to occur or was greatly reduced and no longer interfered with the centrifugation of large particles.

Centrifugation procedures.—Five centrifugation procedures were investigated. Table 2 illustrates these methods in diagrammatic form.

Method I proved to be an unsatisfactory centrifugation procedure, since the suspending medium was incapable of preventing agglutination of cellular particles (Tables 1 and 2) and since the gravity increases were too rapid to permit removal of large particles, tissue debris, and fat prior to high gravity centrifugation. For this reason, the high gravity sediment contained fat, visibly flocculated masses of cellular particles, and tissue debris. A photograph characteristic of the second high gravity sediment of a Hodgkin's lymph node prepared by this method is illustrated in Plate I, Figure 1.

Methods II and III were also unsatisfactory for the reasons mentioned above (Tables 1 and 2) and, in addition, because the initial supernatant fluid containing the majority of cellular particles was discarded during the first step of the centrifugation procedure. Plate I, Figure 2, illustrates agglutinated particles obtained in the 25,000 *g* sediment of a Hodgkin's lymph node prepared by Method II.

Agglutination of cellular particles did not occur in tissue homogenates prepared by Method IV in 0.1 M phosphate buffer, pH 8.0 containing heparin (0.03 per cent or 0.3 per cent)—Tables 1 and 2. The gradual increases in gravity of this method made it possible to sediment cellular particles from the supernatant fluid according to size and to remove them with each centrifugation step. Fat was removed in a similar manner from the surface of the supernatant fluid until no more was present. The resulting high gravity sediment was suitable for visual and chemical study. Plate I, Figures 3 and 4, illustrates the appearance of sedimented particles in electron microscope photographs of high gravity sediments centrifuged in 0.1 M phosphate buffer, pH 8.0 with and without the addition of heparin to the suspending medium.

Method V was adopted in preference to Method IV, since the former makes it possible to wash the high gravity sediment (by resuspending it in fresh diluent and recentrifuging at high gravity) and to complete the procedure in one day. The visual characteristics of the high gravity sediments, prepared by Method V in 0.1 M phosphate buffer, pH 8.0 containing 0.03 per cent heparin and observed in the electron microscope, could not be distinguished from those of sediments prepared by Method IV in the same diluent. To determine the effect of decreasing concentrations of heparin on particle agglutination, lymph node samples were suspended and centrifuged in 0.1 M phosphate buffer, pH 8.0 containing 0.3 per cent, 0.03 per cent, and 0.005 per cent heparin. High gravity sediment samples prepared in buffer with 0.3 per cent and 0.03 per cent heparin added contained dispersed particles; the sediments of those prepared in buffer containing 0.005 per cent heparin exhibited the same degree of particle agglutination observed in the absence of heparin. The low gravity sediments of samples prepared in buffer containing 0.3 per cent heparin contained filamentous mitochondria whose dimensions ranged from 0.1 μ to 0.2 μ in width and from 0.5 μ to 3.0 μ in length. Spherical and oval or slightly elongated mitochondria were also observed in sizes ranging from 0.5 μ to 1.5 μ. Examples of mitochondria observed in the low gravity sediments of a Hodgkin's lymph node preparation are illustrated in Plate I, Figures 5–8.

Preparation of samples for electron microscopy.—Accepted standard procedures were used to coat electron microscope screens with 0.3 per cent Form-

ELECTRON MICROGRAPHS OF CENTRIFUGATION SEDIMENTS

FIG. 1.—Agglutinated particles in the sediment of a Hodgkin's lymph node cell suspension prepared by Method I, following centrifugation at 85,000 *g*. Mag. ×20,000.

FIG. 2.—Agglutinated particles, observed following 25,000 *g* centrifugation, in the sediment of a Hodgkin's lymph node cell suspension prepared by Method II. Mag. ×20,000.

FIG. 3.—Agglutinated particles, observed following 25,000 *g* centrifugation, in the sediment of a suspension prepared by Method IV (without the addition of heparin to the suspending medium). Mag. ×20,000.

FIG. 4.—Dispersed particles, observed following 25,000 *g* centrifugation, in the sediment of a Hodgkin's lymph node cell suspension prepared by Method IV (with the addition of 0.03 per cent heparin). Mag. ×20,000.

FIGS. 5, 6, and 7.—Filamentous mitochondria observed in the low gravity (1,500 and 3,000 *g*) sediments of a Hodgkin's lymph node prepared in 0.1 M phosphate buffer, pH 8.0 containing 0.3 per cent heparin. Mag. ×20,000.

FIG. 8.—Spherical mitochondria observed under the same conditions described in Figures 5, 6, and 7. Mag. ×20,000.

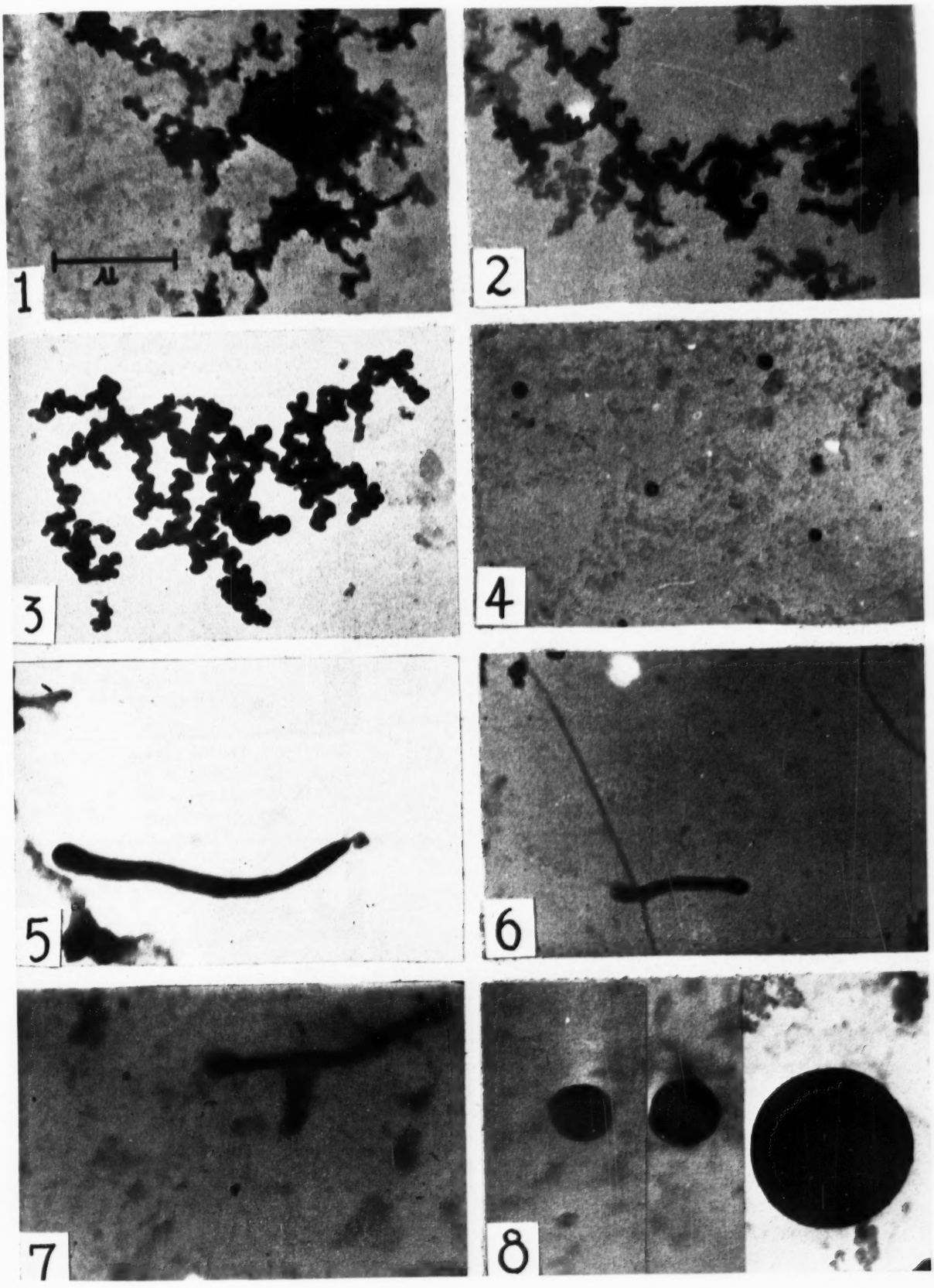
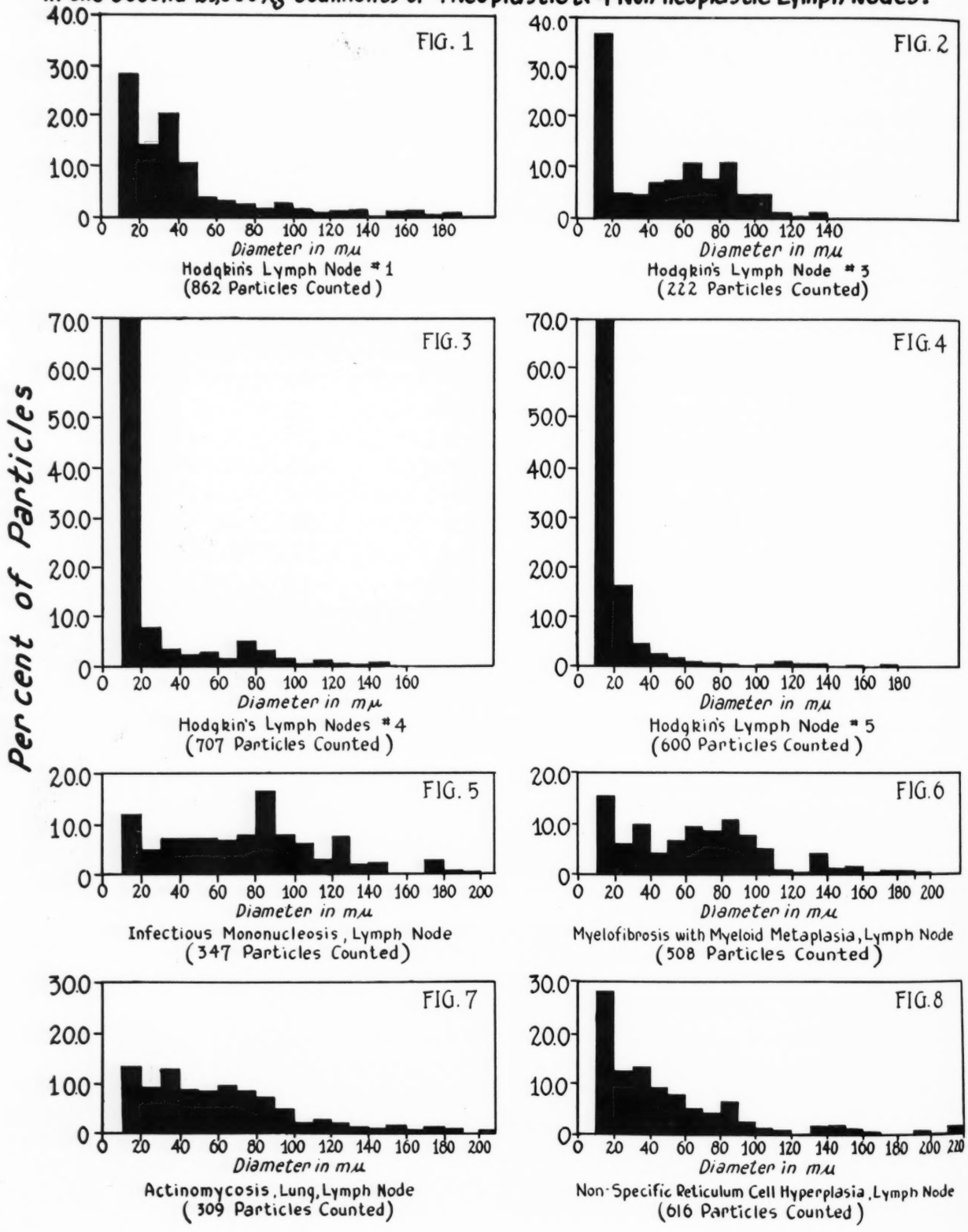


PLATE I, FIGS. 1–8

Histograms Illustrating the Size Distribution of Cellular Particles Obtained in the Second 25,000 xg Sediments of 4 Neoplastic & 4 Non-neoplastic Lymph Nodes.



var (15). The final high gravity centrifugation sediments were resuspended in liquid media and centrifuged for 10 minutes at 1,500 *g*. Formvar-coated electron microscope screens were covered with samples of the supernatant fluids resulting from this procedure. One drop of the centrifugation sample was allowed to remain in contact with the screen for 5 minutes; the remaining fluid was removed by suction. It was observed that flushing the screens with filtered distilled water before the samples had completely dried removed crystals present as a result of evaporation and concentration of the buffer used to suspend the original sample. The dried sample-containing screens were strapped on glass coverslips with Scotch Tape and left in contact with the vapors of 2 per cent osmic acid for 2 hours at room temperature, after which they were washed several times with filtered distilled water.

Control preparations, consisting of screens covered with samples of suspending medium and samples of 0.3 per cent heparin stained with osmic acid, were examined in the electron microscope. These screens did not present an appearance similar to those covered with samples of centrifugation products. To avoid a potential source of contamination, it was necessary to require that no smoking occur in the laboratory in which these preparations were made, since tobacco smoke particles may appear similar to tissue particles under the electron microscope. Glassware was cleaned with great care, including a number of rinses with double-distilled water to insure the elimination of all extraneous material, and filtered double-distilled water was used in the preparation of suspending media.

All preparations were dried over phosphorus pentoxide before exposing them to osmium vapors and before examination in the electron microscope. The photographs illustrated in Plate I were taken at a magnification of 5,000 and 10,000, respectively, and were enlarged to 20,000.

Comparison of neoplastic and non-neoplastic lymph node cellular particles in the electron microscope.—A comparison of particle size distribution in the second 25,000 *g* sediments of seven Hodgkin's, one lymphosarcoma, and eight non-neoplastic lymph nodes prepared by Method V was made. Histograms of four Hodgkin's and four non-neoplastic lymph node samples are illustrated in Plate II, Figures 1–8. All high gravity centrifugation sediments contained polydispersed particles ranging in size from 10 to 280 mμ. Table 3 contains the results of a statistical evaluation of the particles obtained in thirteen lymph node high gravity sediments. The mode or measurement which occurs most frequently is in the 10–20-mμ size range in the Hodgkin's samples; no mode representative of the group is observed in the non-neoplastic samples. The curve skewness is greater and the standard deviation smaller in the Hodgkin's than in the non-neoplastic samples. The standard deviation

TABLE 3

STATISTICAL EVALUATION OF PARTICLE SIZE DISTRIBUTION OBTAINED IN HIGH GRAVITY (25,000 *g*) CENTRIFUGATION SEDIMENTS OF NEOPLASTIC AND NON-NEOPLASTIC LYMPH NODES

Sample	Mean particle size (mμ)	Mode (mμ)	Skewness	Standard deviation
Hodgkin's #1 (Plate II, Fig. 1)	44.70	16.74	+0.83	39.67
Hodgkin's #3 (Plate II, Fig. 2)	48.50	15.40	+0.32	32.41
Hodgkin's #4 (Plate II, Fig. 3)	28.49	15.29	+1.27	26.95
Hodgkin's #5 (Plate II, Fig. 4)	22.60	15.65	+0.87	18.92
Hodgkin's #6	45.40	23.24	+0.51	28.03
Hodgkin's #7	37.60	15.40	+1.45	32.48
Lymphosarcoma	40.20	24.69	+0.97	33.36
Infectious mononucleosis (Plate II, Fig. 5)	63.10	85.00	−1.02	42.56
Cerebral hemorrhage	56.04	33.33	+1.01	57.93
Myelofibrosis with myeloid metaplasia (Plate II, Fig. 6)	67.11	16.26	+0.11	40.90
Actinomycosis of lung (Plate II, Fig. 7)	65.02	17.74	+0.56	42.69
Nonspecific reticulum-cell hyperplasia (Plate II, Fig. 8)	52.50	16.39	+0.98	45.21
Accidental death	77.21	74.23	+0.30	45.37

of the lymphosarcoma sample studied resembles the Hodgkin's samples.

Chemical and spectrophotometric studies.—Qualitative chemical determinations involving centrifugation products of neoplastic and non-neoplastic lymph nodes indicated the presence of ribose, desoxyribose, and purine bases in the low gravity (1,500 and 3,000 *g*) sediments and in the supernatant fluid obtained following the first 25,000 *g* centrifugation. Similar studies established the presence of ribose and purine bases and the absence of desoxyribose in the washed second 25,000 *g* sediments.

The acid hydrolysates (5 per cent hydrochloric acid) of the first 25,000 *g* supernatant fluid and the second 25,000 *g* sediment of seven Hodgkin's, one lymphosarcoma, and five non-neoplastic lymph nodes were subjected to quantitative spectrophotometric analysis for nucleic acid (assuming that the extinction coefficient of pure ribose nucleic acid is 21.3 l. gm.$^{-1}$ cm.$^{-1}$ at 260 mμ). Table 4 contains the results of these determinations.

TABLE 4

PER CENT NUCLEIC ACID IN FRACTIONS OF NEOPLASTIC AND NON-NEOPLASTIC LYMPH NODES

Fraction	Hodgkin's	Lympho-sarcoma	Non-neoplastic
Supernatant fluid	7.1–15.1	21.8	9.1–18.7
Sediment	2.2– 6.1	4.6	4.1–10.6

DISCUSSION

Since the principal problem encountered under the conditions of the present experiment involves the segregation of intact and dispersed cellular particles by centrifugation, an attempt was made to determine the optimum physical and chemical conditions under which this might be accomplished. Accordingly, consideration was given to the hydrogen ion concentration and ionic strength of the suspending medium, the presence of agents capable of preventing gel formation and agglutination of cells and their particulate components, and the type of centrifugation procedure necessary to retain the maximum number of particles smaller than 350 mμ in the high gravity sediment. Claude (4) demonstrated that agglutination of cellular particles occurs in physiologic saline when the hydrogen ion concentration of rat tumor cell homogenates is not maintained above pH 7.0. Lazarow (12) and Hogeboom *et al.* (11) were unable to maintain the dispersion of cellular particles suspended in solutions of a variety of electrolytes in which the type of buffer, ionic strength, and hydrogen ion concentration were varied. In the case of human lymph node homogenates, dark field examination has confirmed these observations, using a number of buffered and unbuffered electrolytes as tissue-suspending media. As noted above, the use of 0.1 M phosphate buffer pH 8.0, containing 0.03 per cent heparin, does not cause agglutination of particles and thus insures the dispersion of small particles in the absence of sucrose and in the presence of a buffered electrolyte. Since the same medium containing 0.005 per cent heparin, isotonic phosphate buffer pH 7.0 containing 0.3 per cent heparin, and isotonic saline containing 0.3 per cent heparin do not prevent agglutination of particles under the same conditions of extraction and centrifugation, it must be concluded that the effective pH and concentration range of heparin under these conditions is narrow.

Hogeboom *et al.* (11) report that elongated mitochondria become spherical when liver cells are broken up in isotonic sucrose and in isotonic salt solutions but retain their original shape and ability to take up Janus Green B dye when broken up in hypertonic (0.88 M) sucrose. The phenomenon of enspherulation of mitochondria in salt solutions has been observed by a number of workers, including Hoerr (10), Zollinger (14), and Dalton *et al.* (6). This phenomenon occurs both with cells, suspended in salt solution, which remain unbroken, and with suspensions of disrupted cells. Although rod-shaped, oblong, and spherical, Janus Green B dye-containing mitochondria, observed in the light microscope in intact lymph node cells, appear similar in size and shape to particulate elements observed outside the cell in the low gravity sediments of tissue suspensions following centrifugation in 0.1 M phosphate buffer pH 8.0 containing heparin, it has not been proved that these particles are resistant to fragmentation.

The observation that particles ranging from 10 to 280 mμ are found in the high gravity sediments of neoplastic and non-neoplastic lymph node suspensions on electron microscope examination indicates that, under the conditions of the present experiment, the small particle population is polydispersed. The predominance of particles approximately 10–20 mμ in the Hodgkin's lymph node sediments and the lack of a predominant size range in the non-neoplastic lymph node sediments are statistically significant. Since Dalton *et al.* (6) have reported that the mitochondria of certain mouse tumor cells are more fragile than the mitochondria of the normal hepatic cell, one may inquire whether the difference between Hodgkin's and non-neoplastic particle size distribution is due to fragmentation of mitochondria. Further study relating to this problem is in progress.

Although Hogeboom *et al.* (11), using 0.25 M and 0.88 M sucrose, have described the successful segregation of rat liver cell mitochondria and microsomes without agglutination of particles, occasional particle clumps and occasional large particles have been observed in the present experiment in the high gravity sediments of tissues suspended in sucrose. On electron microscope examination, although detectable clumping of particles is present in sediments resuspended following centrifugation in all of the media mentioned in this discussion, the results obtained using heparinized buffer appear more satisfactory than those obtained using sucrose and other diluents referred to above.

The presence of fat in the tissue suspension constitutes an added problem encountered when human lymph nodes are used as a source of cell particles. Unless the fat is allowed to migrate centripetally and is removed following each of a series of low gravity centrifugations until no more is present, it is sedimented in association with cell particles at high gravity and resuspension of these particles is no longer possible. The multiple-step centrifugation procedure (Methods IV and V), devised in part as a result of the need for removing fat, has proved satisfactory for this purpose.

Cohen (5) reported that heparin in small amounts does not replace the nucleic acid of the tobacco mosaic virus and that precipitation of the virus by means of heparin does not seem to involve a chemical combination of the two, since the virus-heparin preparation exhibits, on centrifugation in an optical ultracentrifuge, the same double boundary, sedimentation constants, and electron microscope appearance as the original suspension in the absence of heparin. Since, on electron microscopy and dark field examination, particulate cellular components obtained from lymph node cells broken up in heparinized buffer appear similar to those seen in intact cells in the absence of heparin, it is suggested that no readily detectable changes in the size and shape of these particles occur as a result of heparin.

Anderson and Wilbur (2) reported that heparin causes the release of nucleic acid from rat liver nuclear and mitochondrial cell components and suggested that this compound may displace the nucleic acids from the basic proteins of these particulates due to its strongly acidic sulfuric acid prosthetic groups. Although the amount of nucleic acid recorded in the high gravity sediments (in the present study) is in agreement with that reported by Abrams and Cohen (1) in the small particle fraction of human tonsillar lymphoid tissue, the relatively large amount found in the high gravity supernatant fluid may indicate a heparin-induced release of nucleic acid from one or more of the cell particulate components. Studies to determine the relation of the concentration of nucleic acid in the high gravity supernatant fluid to the concentration of heparin present in the tissue diluent are in progress.

The type of nucleic acid present both in the high gravity sediments and in sediments obtained at lower gravitational levels was investigated to determine whether these sediments contained fragmented nuclear constituents. The presence of ribose and the absence of desoxyribose in the high gravity sediments suggest that the latter was removed prior to high gravity centrifugation.

SUMMARY

The segregation of dispersed cellular particles obtained from human lymph nodes has been carried out using a ten-step centrifugation procedure and a fluid medium consisting of 0.1 M phosphate buffer, pH 8.0, containing 0.03 per cent or 0.3 per cent heparin. Nuclear, mitochondrial, and submicroscopic particle fractions are obtained at suitable gravitational forces.

An analysis of the particle populations of the high gravity centrifugation sediments indicates the presence of polydispersed particles ranging from 10 to 280 mμ in neoplastic and non-neoplastic lymph nodes. The predominant particle size obtained from Hodgkin's lymph nodes, 10–20 mμ, differs significantly from the particle sizes found in the non-neoplastic lymph nodes.

The presence of ribose nucleic acid and the absence of desoxyribose nucleic acid was demonstrated in the high gravity sediments. The per cent nucleic acid in high gravity sediments of neoplastic and non-neoplastic lymph nodes were compared.

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Tobacco Smoking Habits and Cancer of the Mouth and Respiratory System

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Those concerned with the general aspects of disease trends have been much disturbed by the sharp rise in the frequency of lung cancer in recent decades. This concern seems fully justifiable, for this once rare disease now causes almost as many deaths as pneumonia or tuberculosis among white males in a city like Chicago (2). Of all neoplastic diseases in adult males, the incidence of lung cancer has shown the most rapid increase in recent years. Its incidence has been shown (2) to be greatest in the dirtier sections of industrial cities, where pneumonia and tuberculosis rates are also highest. That these high rates should involve the men so much more than the women of the same districts suggests that some factor or factors other than outdoor air pollution must be involved.

Carcinogenic compounds have been isolated from the tarry substances of both coal and tobacco smoke, as well as from the smoke of a great variety of other slowly burning organic substances. These tarry substances are otherwise irritating to the respiratory passages of laboratory animals inhaling tar-laden soot (5). The three serious respiratory diseases (cancer, pneumonia, and tuberculosis) show such similar variation in incidence with increasing density of air pollution in our industrial cities that one is led to suspect long-standing, chronic irritation as the responsible factor.

In searching for possible reasons why men should be so much more involved than women in the sharp rise of respiratory disease death rates in industrial districts, we deemed it wise to investigate the possible contributory role of tobacco smoking. We felt that milder stages of irritation from either tobacco smoke or coal smoke alone might reach dangerous degrees of damage when the two were summated. Having previously shown a significant relationship to exist between air pollution and the three respiratory disease death rates (2), we next investigated their connection with tobacco smoking habits. In the present report dealing with the smoking habits of some 568 white men dying of buccal and respiratory tract cancer in Detroit and Cincinnati, we show a significantly higher incidence of pipe and cigar smoking among the buccal cancer victims than among controls of similar sex and age distribution and a significantly higher incidence of all forms of smoking among those dying of lung cancer.

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METHODS

Smoking habits in a control population.—Columbus, Ohio, with a white population of 270,183 in the 1940 Census, was chosen as a representative American city of moderate size and industrialization. House-to-house visits were made (in 1947) in each of the city's 61 census tracts until 0.9 per cent of the calculated number of white males in each had been interviewed; smoking data were obtained only on residents 20 years of age or over. Although our original data contain detailed information on amount as well as type of smoking, Table 1 gives only three categories: (*a*) cigarettes, (*b*) pipe or cigars or any smoking combination containing either, and (*c*) nonsmokers.

In addition to the smoking habits by age, our survey also included a division of the whole city into clean, intermediate, and dirty districts. Of the 61 total census tracts, 1–10, 25–27, 37, and 54 were listed as the cleanest; tracts 11–20, 44–49, 55, 56, and 58–61 were called intermediate; and tracts 21–24, 28–36, 38–43, 50–53, and 57 comprised the areas dirtiest in air pollution.

From the data calculated on a percentage basis, it was found that cigarette smoking was heaviest among younger male adults and decreased with advancing age, while the reverse was true with cigar and pipe smoking. Cigarette smoking was also heaviest in the dirty (poorer) sections of the city and diminished toward the cleaner periphery, while the reverse was again true for pipe and cigar smoking. The higher cost of cigars may well have been a factor in explaining this smoking pattern that shifted with varying economic status, while the higher percentages of cigarette smokers in the younger age groups probably represented the

rapidly increasing adoption of this form of smoking since World War I.

Since objections might be raised against the use of Columbus smoking statistics as control data for comparison with various disease groups in other cities, we also ran an additional check on one category of persons in Cincinnati. Among 310 20–29-year-old white women scattered in all census tracts of Columbus, 39.68 per cent were cigarette

TABLE 1

TOBACCO SMOKING HABITS (BY AGE) OF WHITE MEN IN COLUMBUS, OHIO (1947)

	Age group (years) (20–29)		(30–39)		(40–49)		(50–59)		(60–69)		(70–79)		(80+)	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
Cigarettes	233	66±2.5*	116	61±3.5	131	61±3.3	97	52±3.7	41	34±4.5	9	13±4.2	1	5±5.4
Pipe, cigars, or combination	38	11±1.7	32	17±2.7	42	19±2.7	45	24±3.1	37	31±4.3	21	30±5.7	9	47±11.8
Nonsmokers	80	23±2.2	42	22±3.0	43	20±2.7	46	24±3.3	42	35±4.5	39	57±6.1	9	47±11.8
TOTALS:	351		190		216		188		120		69		19	

* Standard error, calculated according to the formula ($s = \sqrt{pq/N}$) where p in this case represents the per cent smoking cigarettes, q the per cent not smoking cigarettes, and N the total number in that age group (smoking cigarettes and not smoking cigarettes) (3).

TABLE 2

TOBACCO SMOKING HABITS IN VICTIMS OF RESPIRATORY TRACT AND BUCCAL CANCER*

Group	Total group (No.)	(Mean age)	Cigarettes only (No.)	(Per cent)	(Standard error)	Pipe, cigars, or combinations (No.)	(Per cent)	(Standard error)	Nonsmokers (No.)	(Per cent)	(Standard error)
Buccal (No. 45)	124	65.4	44	35	±4.3	68	55	±4.5	12	10	±2.7
Columbus controls	185	65.3	60	32	±3.4	55	30	±3.4	70	38	±3.6
Diff./standard error of diff.†				3.0/±5.5=0.5			25/±5.6=4.5			28/±4.5=6.2	
Respiratory (No. 47)	444	58.3	246	55	±2.4	166	37	±2.3	32	7	±1.2
Columbus controls	430	58.2	187	43	±2.4	112	26	±2.1	131	31	±2.2
Diff/standard error of diff.				12/±3.4=3.5			11/±3.1=3.6			24/±2.5=9.6	

* Detroit and Cincinnati white males.

† Standard error of the percentage difference (3) calculated thus: $s = \sqrt{(p_1q_1/N_1) + (p_2q_2/N_2)}$.

smokers, as compared to 41.35 per cent smokers among 520 white women of similar age and city-wide distribution over Cincinnati. This difference is insignificant.

In making a comparison of Columbus smoking habits with those of Detroit and Cincinnati respiratory and buccal cancer victims, it was considered wise to use the "whole city" statistics rather than those of any one economic or regional group in Columbus. Cancer victims of Cincinnati and Detroit were scattered throughout the city in all socio-economic groups. The only correction deemed necessary was one of age distribution (as indicated later).

Smoking habits of male victims of buccal and respiratory tract cancer as compared to those of control groups.—Names, addresses, and names of next-of-kin or informant specified on the death certificate were obtained for all people dying of cancer of the respiratory tract and of the buccal and pharyngeal tissue (Nos. 47 and 45, International List of Causes of Death) for the years 1940–45, inclusive, in Cincinnati, and 1942–46, inclusive, in Detroit. Positive information on smoking habits was obtained on 254 Cincinnati cases and on 503 in Detroit either by circular letter or by direct visitation to the next-of-kin or specified informant. This represented positive information in approximately 50 per cent of the deaths listed. Only with white males were numbers of cases adequate for statistical stability (175 in Cincinnati and 393 in Detroit).

Table 2 shows the smoking habits of the Cincinnati and Detroit white male respiratory tract and buccal cancer victims, as compared to Columbus controls of similar color, sex, and age distribution. The data on the Columbus control groups is obtained from Table 1 and is so presented that the percentage of the total for each decade is identical with the cancer group with which it is compared. Control groups were made as large as possible, the limiting factor usually being the number of cases

available in one of the more advanced age decades. Single control individuals were not selected to make up the total; this was accomplished by taking the required fractional part of the age decade's total of cigarette smokers, pipe or cigar users, and nonsmokers. Percentage age distribution of the two groups of cancer victims is presented in Table 3.

TABLE 3

AGE DISTRIBUTION OF THE DETROIT AND CINCINNATI CANCER VICTIMS

Age group (years)	Resp. tract cancers (No. 47) (No.)	(Per cent)	Buccal cancers (No. 45) (No.)	(Per cent)
10–19	1	0.2	0	0.0
20–29	2	0.4	2	1.6
30–39	16	3.6	1	0.8
40–49	72	16.2	10	8.1
50–59	165	37.2	21	16.9
60–69	127	28.6	48	38.7
70–79	51	11.5	29	23.4
80+	10	2.3	13	10.5
TOTAL:	444	100.0	124	100.0

Table 2 illustrates that Cincinnati and Detroit white males dying of buccal cancer show no significant difference in cigarette smoking habits from the Columbus control group. There is a decided and highly significant increase in pipe and/or cigar usage among buccal cancer victims, however.

Cincinnati and Detroit victims of cancers of the respiratory tract (No. 47), on the other hand, exhibit highly significant elevations above normal in both cigarette and pipe and/or cigar usage. With both the buccal cancer group and respiratory tract cancer group, the incidence of nonsmokers is only one-fourth as great as among the proper Columbus controls. These differences in percentages of nonsmokers are highly significant.

While our data on smoking habits were fairly specific as to the amount of tobacco smoked daily, they were not satisfactorily complete as to duration in years. Comparison was made between Columbus controls and one cancer group (respiratory tract cancer victims in Detroit) as to the percentage of "heavy" smokers among the cigarette users.

PERCENTAGE OF "HEAVY" SMOKERS AMONG CIGARETTE USERS

	30–39 yrs.	40–49 yrs.	50–59 yrs.	60–69 yrs.	70–79 yrs.
	(Per cent)				
Detroit cancer victims	86	79	87	73	67
Columbus controls	87	88	86	82	67

Here no significant difference is seen in the percentage of cigarette users who smoke one pack or more a day ("heavy" smokers).

DISCUSSION

From the findings here presented, it is evident that buccal cancer victims are significantly more addicted to pipe and/or cigar smoking than are the proper control population groups. Buccal cancer and control groups show no significant difference in cigarette smoking habits. This association of pipe and/or cigar usage with cancers of the buccal tissues has often been noted in medical literature and has been attributed to the more sluggish combustion and greater production of irritating tarry materials in these forms of smoking.

With cancers of the respiratory tract from the larynx downward, an abnormally high percentage of cigarette smokers, as well as of pipe and/or cigar users, is found. This group of cancer victims exhibits significantly increased percentages in all forms of smoking.

Roffo (3) found that 95 per cent of all respiratory tract cancers occurred in smokers. In our series, 93 per cent of all lower tract cancer victims, but only 90 per cent of upper tract cancer victims, were smokers. Many other investigators have reported 90–95 per cent incidence of tobacco smoking among respiratory tract cancer victims, with rather direct indictment of cigar and pipe smoking for cancers of the lip and tongue. Ours is the first study to indicate an indictment of all forms of smoking for cancers of the lower respiratory tract.

Schrek *et al.* in their recent paper (4) claim to have found a significant correlation between heavy cigarette smoking and respiratory tract cancers (from the pharynx down) and a negative correlation with cigar and pipe smoking, except for cancers of the lip. Their conclusions may be doubtful, however, because of their use of hospitalized tumor patients as their control group instead of a normal population cross-section, and because their data fail to indicate in any way the classification of the large percentage of both control and respiratory tract cancer individuals who were "combination" smokers—smoking cigarettes *and* pipe or cigars. Since Schrek *et al.* frankly stated that their interest centered on cigarette smoking, it might perhaps be assumed that any such "combination" smokers were listed only as cigarette users.

In these investigations we made correlation studies both ways, with "combination" smokers in control and cancer groups listed first as cigar and pipe smokers and then as cigarette smokers. In both cases the correlation values were similar. Almost 10 per cent of the Columbus men we interviewed were such "combination" smokers, these constituting almost half of the pipe and cigar smokers.

CONCLUSIONS

1. The percentage of cigar and pipe smokers is almost twice as high among white male victims of buccal cancer as among appropriately selected controls; all forms of smoking are significantly higher among victims of respiratory tract cancer than among the controls.

2. The percentage of nonsmokers among white male respiratory tract and buccal cancer victims is only one-fourth as high as among properly selected control groups.

3. Cigarette smoking seems to bear a highly significant relation to cancers of the respiratory tract but no significant relation to the incidence of buccal cancer.

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The Use of Acetamide in the Meiostagmin Reaction

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The original meiostagmin reaction (MR) was based on the reduction of the surface tension of cancer serum after incubation with tumor extracts (1–4). The numerous variations and modifications of this method have been reviewed by Stern and Willheim (21). The MR shares the lack of specificity with other lability tests; but, apart from pregnancy, only very few noncancerous conditions are known to interfere, and these are readily diagnosed by clinical methods and auxiliary technics of the MR (14, 23). Keller and Künzel (11) used Lecomte du Noüy's dynamic principle (13) to observe surface tension variations in serum-sodium oleate systems. Significant differences between cancer and normal sera were noted. The surface tension buffering power of sera against strong surface-active reagents has been investigated in connection with the MR, but buffering in the presence of reagents expected to raise the surface tension of cancer sera has not been investigated so far. The present work describes experiments of the latter kind. Keller and Künzel explained their results in terms of a combination of soap and serum proteins. More recent work (5) supports such views and indicates that similar combinations may take place between amides and serum proteins. For reasons of simplicity the effects of acetamide, urea, and diacetamide (15) were tested in our experiments. The first two are denaturing agents, the former being weaker than the latter (20). The denaturing effect of diacetamide is the same as that of acetamide, judging from the titration of various proteins in isotonic aqueous saline with absolute alcohol, using Keller's method (10) with nephelometric standards.

METHODS

Cancer and normal sera were taken at random at the Royal Hobart Hospital, Launceston General Hospital, and Sydney Hospital in 5–10-ml. lots under sterile conditions. Oxalated and citrated sera were found unsuitable. The samples were defibrinated at the sampling stations. Different methods of defibrination did not affect the results, nor did the use of slightly hemolysed samples, contrary to observations with an early form of the MR (19).

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The effects of delays in testing are shown in Table 3. Centrifuging immediately after sampling gave better quantitative results. Sodium cyanide helps to counteract the effects of a delay in testing, but it was not used in the reported experiments. Sodium fluoride has no effect and copper sulfate an adverse effect.

The sera were diluted with 5 per cent aqueous solutions of the amides. With lower concentrations the results became inaccurate, and higher concentrations were avoided to minimize denaturing effects. The amide solutions were added to the sera up to 512 dilutions. The solutions were homogenized by gently tilting the stoppered test tubes 10 times, pouring into standard watch glasses of 4-ml. capacity with a surface: volume ratio of 4.0, and allowing the solutions to come to equilibrium. The latter process required a few minutes and 3–4 preliminary measurements. The surface tensions (γ) of the original and diluted sera were measured at 18° C. in a thermostatically controlled room with a Cambridge Du Noüy Tensiometer. The average of 3–6 measurements was taken as the correct value as soon as the measurements became constant within $\pm$ 0.1 dyne/cm. Slightly fermented sera have low surface tensions in the undiluted state (γ = 49–52 dyne/cm) and were discarded.

RESULTS AND DISCUSSION

With urea (γ = 73.0 dyne/cm in 5 per cent solution), no significant effect could be observed. Since weaker denaturing agents such as acetamide and diacetamide gave more pronounced effects, these must be due mainly to causes other than denaturation. No consistent results could be obtained with diacetamide (γ = 67.8 dyne/cm in 5 per cent solution) other than a sharp initial drop of the surface tension at low dilutions (7).

With acetamide purified by distillation alone, curves of different shapes could be obtained for cancer and normal sera, but the findings could not be duplicated with accuracy. The effect is ascribed to the odorous impurity of acetamide which is absent when acetamide is repeatedly recrystal-

lized from benzene and ethyl acetate and washed with ether. Acetamide solutions keep unchanged for 4 hours, but inaccurate results are obtained with solutions 24–30 hours old.

With fresh 5 per cent solutions of acetamide ($\gamma = 70.1$ dyne/cm), the surface tension versus dilution curves rise to a maximum at 16–50 dilutions. At further dilutions transient cloudiness appears, and the surface tension values fall to a minimum at 100–200 dilutions. This minimum value ($\gamma_{min.}$) is lower than the surface tension of the undiluted serum (γ_0) with cancer, pregnancy, and some other sera. Since the rise to higher surface tension values past the minimum may be slow or fast, it was attempted to characterize the general behavior of the curves by a conventional measure termed "critical area" (CA). A positive CA is defined as the area inclosed by the curve and a straight line drawn through γ_0 parallel with the dilution axis. If this line is a tangent of the curve at its minimum, the CA is reported as 0. Otherwise, the CA is reported as negative (−). In sera exhibiting powerful buffering effects around the value of $\gamma_{min.}$ it may happen that 512 dilutions are not sufficient to circumscribe the positive CA. In a few cases of this kind the area was completed by a line drawn perpendicularly through the point corresponding to 512 dilutions on the abscissa. A positive CA may be evaluated by counting squares or by planimetry. Figures reported in Tables 1, 2, 3 and 4 have been rounded off to the nearest ten with an average error of about 10 units. Examples of typical cancer (C41S) and noncancer (N11) curves are shown in Figure 1.

TABLE 1

RESULTS OBTAINED ON CANCER SERA

Number	Sex	Age	Diagnosis	Treatment	γ_0	γ_{min}	G	CA
C 1	F	60	C., cervix of uterus		58.0	56.0	2.0	320
C 2	M	54	C., stomach		58.0	54.2	3.8	950
C 3	F	44	C., cervix of uterus		57.0	55.0	2.0	370
C 4	F	62	C., uterus		55.0	53.8	1.2	80
C 5	F	35	C., ovaries	morphia	57.2	53.4	3.8	910
C 6	F	75	C., cervix of uterus		57.3	53.8	3.5	210
C 7	M	61	C., lower bowel	O(5 d.)	59.0	57.9	1.1	90
C 8	F	58	C., lower bowel	O(2 w.)	58.5	57.8	0.7	50
C 9	M	66	C., head of pancreas	O	58.2	55.9	2.3	520
C 10	F	62	C., breast, metastases	O(1 w.)	57.0	55.3	1.7	370
C 11	M	57	C., stomach, "	morphia	57.0	55.6	1.4	320
C 12	M	62	C., stomach	O, penicillin	57.5	57.1	0.4	30
C 13S	F	68	C., breast	O	56.0	55.2	0.8	60
C 14S	F	40	C., cervix of uterus		58.9	57.3	1.6	210
C 15S	M	63	Squam. carc. ani		55.3	54.3	1.0	290
C 16S	M	51	Carc. piriform fossa		56.9	54.6	2.3	380
C 17S	M	59	Squam. carc., hypopharynx		56.2	55.7	0.5	30
C 18L	F	49	Carc., cheek	X, O(7 m.)	57.4	54.8	2.6	650
C 19L	M	45	Astrocytoma pariet.	X, PB	57.8	56.7	1.1	70
C 20L	M	49	Carc., larynx	X	58.9	57.9	1.0	270
C 21L	F	63	Carc., breast	X, PB	58.0	55.3	2.7	490
C 22L	F	74	Carc., tongue	X	58.0	57.1	0.9	100
C 23L	F	47	Astrocytoma pariet.	X, PB	57.7	56.8	0.9	130
C 24	F	59	Endocervic. carc., II		59.5	57.8	1.7	190
C 25S	M	36	Carc., bladder	O(11 m.)	57.0	56.7	0.3	10
C 26S	M	62	Squam. carc., lip		58.4	57.4	1.0	80
C 27L	M	72	Carc., jaw, 7 years	X	57.8	56.7	1.1	210
C 28L	F	38	Pituitary tumor	X	57.4	56.6	0.8	110
C 29L	F	76	Squam. carc., temple	X	56.5	54.8	1.7	280
C 30L	F	55	Adenocarc., uterus		56.7	56.0	0.7	80
C 31S	F	68	Carc., cervix, II		57.8	57.2	0.6	50
C 32S	F	39	Carc., cervix, II		58.8	57.7	1.1	150
C 33S	F	54	Scirrh. carc., breast	O(4 m.)	56.0	56.0	0.0	0
C 34L	F	71	Carc., breast, 22 years		57.0	55.8	1.2	100
C 35L	F	38	Epithelioma, nose		58.0	56.7	1.3	230
C 36L	F	66	Carc., breast		57.1	55.9	1.2	260
C 37L	M	52	Carc., neck and chin	X	56.4	57.0	−0.6	
C 38S	F	35	Carc., cervix, II	radiotherapy	57.0	56.0	1.0	190
C 39S	M	60	Obstruction of esophagus, anaplast. carc.		56.3	55.8	0.5	40
C 40S	M	52	Malignant angioma	radiotherapy	56.8	56.0	0.8	70
C 41S	M	60	Carc., lip		57.2	55.3	1.9	230
C 42	F	62	C., breast	O, B(4 d.)	56.8	55.0	1.8	230
C 43	M	60	C., pancreas	O(1 w.), B(1 d.)	54.9	55.5	−0.6	
C 44	M	48	C., esophagus	B(2 d.)	53.0	56.2	−3.2	

L: samples from Launceston; S: samples from Sydney; unmarked samples from Hobart; O: operated; B: transfusion of 2 pints of blood; d: days; w: weeks; m: months of operation or transfusion before test; X: X-ray treatment; PB: phenobarbitol.

The use of CA values has the statistical disadvantage of unsymmetrical representations, but CA values are correlated with the symmetrical expression $G = \gamma_0 - \gamma_{min}$. Results on cancer sera are shown in Table 1 and on other sera in Table 2. An analysis of CA values by anatomical sites indicates that great variations are not to be expected (Table 4).

Cases C42, C43, and C44 show the effects of blood transfusion. The CA values in the latter two cases are not comparable with the rest of the data. In the normal series no clinical data were available on Cases N4 and N15. Radiologically treated cases appear to be correlated with flat, elongated curves. It is reasonable to assume that the low CA of C37L is a consequence of successful radiological treatment, but the same could not be said about C12—a case of carcinoma of the stomach treated with penicillin.

Excluding cases C43 and C44 in the cancer series and those of pregnancy, one obtains average CA values (rounded off to the nearest ten) of 220 for 41 cancer cases and of 40 for 11 noncancer cases with positive or zero CA values. Better percentages could be reported by omitting postoperative or radiologically treated cases with low CA values. The average values of G (rounded off to the first decimal) are 2.5 for 4 pregnancy cases, 1.4 for 42 cancer, and −0.8 for 27 noncancer cases. Only one noncancer and nonpregnancy case (4 per cent) exceeds the average G value for cancer, and all cancer cases have G values above the noncancer and nonpregnancy average. A histogram of the G values, shown in Figure 2, illustrates the difference between the results obtained in the cancer and

TABLE 2

RESULTS OBTAINED ON OTHER SERA

Number	Sex	Age	Condition	γ_0	γ_{min}	G	CA
N 1L	M	65	Gastr. hyperacidity	57.2	57.1	−0.1	0
N 2	M	25		55.0	56.3	−1.3	
N 3	F	25		55.2	55.5	−0.3	
N 4	F	20	?	57.8	56.8	1.0	80
N 5	M	24		58.0	57.5	0.5	30
N 6	M	26		58.0	58.9	−0.9	
N 7	M	21		58.6	59.0	−0.4	
N 8	M	39		52.0	54.7	−2.7	
N 9	M	18		57.0	56.5	0.5	20
N 10	M	23		56.5	57.1	−0.6	
N 11	M	24		56.1	58.0	−1.9	
N 12	M	42	Fract. tibia	55.0	56.7	−1.7	
N 13	F	31		56.0	59.2	−3.2	
N 14	M	30	Traum. hydrocele	57.8	57.8	0.0	0
N 15	F	62	Arthritis (?)	57.8	56.8	1.0	70
N 16	F	45	Disloc. ankle	57.0	57.0	0.0	0
N 17	M	35		58.0	58.0	0.0	0
N 18	M	27	Acute lumbago	56.8	58.2	−1.4	
N 19	F	61		56.0	57.5	−1.5	
D 20	F	69	10 years	57.3	57.4	−0.1	0
D 21	M	70		57.0	55.4	1.6	150
D 22	F	76	Cardiac failure	58.4	58.2	0.2	20
D 23L	F	66	3 months	60.2	61.4	−1.2	
D 24L	M	67	3 years	59.0	60.4	−1.4	
D 25L	F	56	2 years	56.8	60.1	−3.3	
D 26L	F	68	11 years	56.4	59.4	−3.0	
D 27L	F	47	14 years	54.0	?	?	
P 28	F	37	37 weeks	56.3	54.1	2.2	380
P 29	F	21	38 weeks	57.0	54.4	2.6	350
P 30	F	22	35 weeks	56.8	54.6	2.2	190
P 31	F	27	24 weeks, twins	57.8	55.0	2.8	500

N: normal; D: diabetes; P: pregnancy; L: samples from Launceston; unmarked samples from Hobart. Treatment: 1L X-ray; 20, 22 insulin; 13 pentothal; 18 sodium salicylate; 20 phenobarbitol.

TABLE 3

EFFECT OF DELAY ON TESTING SERA

Origin	Delay	Average CA	Average G
Hobart	0	320	2.0
Launceston	24 hours	210	1.1
Sydney	48 hours	130	0.9

TABLE 4

ANALYSIS OF CA VALUES BY ANATOMICAL SITES

	Cases	Average CA	Average G
Stomach, esophagus	4	330	1.5
Uterus, ovaries	11	250	1.7
Breast	7	220	1.3
Neck, throat, mouth	9	220	1.0
Head	4	180	1.3
Lower intestine	3	180	0.9

noncancer series (the latter excluding cases of pregnancy). The difference between the corrected standard deviations for G in the cancer and noncancer series is 0.38 ± 0.19. A better indication of the significance of the MR is obtained from the

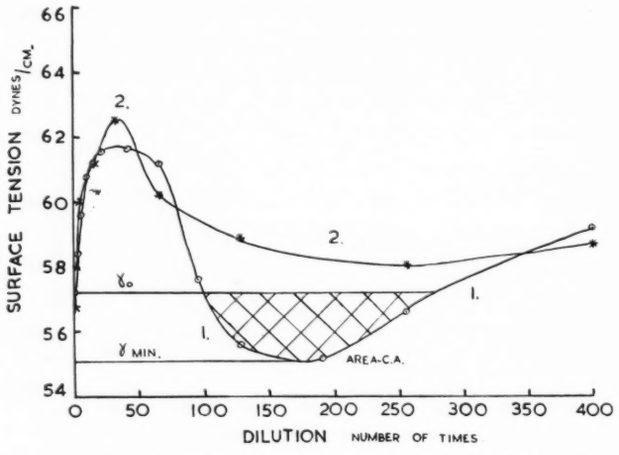

FIG. 1.—Surface tension of cancer (1) and normal (2) serum on dilution with 5 per cent acetamide.

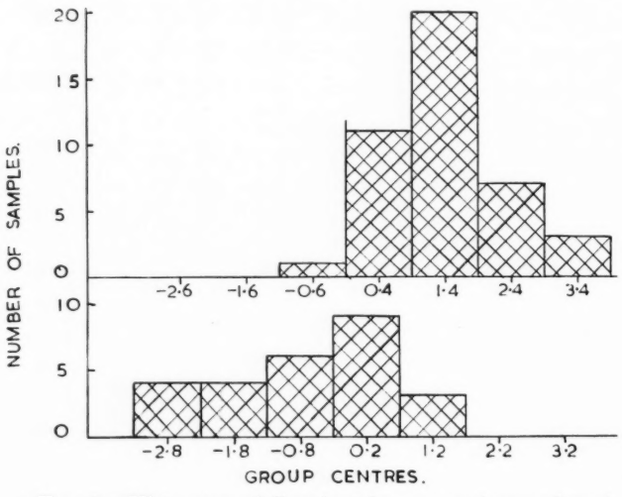

FIG. 2.—Histogram of G values for cancer (upper figure) and noncancer (lower figure) cases. The latter exclude cases of pregnancy.

t-test. For the calculated 8.0 for *t* and 66 degrees of freedom, a probability percentage point of well below 0.1 per cent is obtained. A variance test gives F(25, 41) = 2.0, corresponding to a probability percentage point of about 3. The significance of the CA values in the cancer and noncancer series can be estimated by the χ^2-test. Dividing the samples into cancer and noncancer classes and classes with positive and negative (including zero) CA values, χ^2 is obtained as 39.5, which, for 1 degree of freedom, corresponds to a probability percentage point of well below 0.1 per cent. Almost the same result is obtained if the χ^2-test is applied to the G values with classes comprising positive and negative (the latter including zero) G values.

The effect of dilution with buffer solutions has been studied in the case of cancer, diabetes, and normal sera. Figures 3 and 4 show the results of a

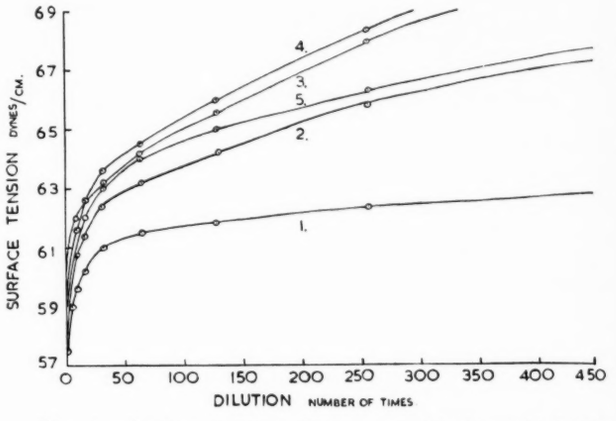

FIG. 3.—Surface tension of serum on dilution with buffer solutions. 1: pH 6.00; 2: pH 6.72; 3: pH 7.30; 4: pH 7.72; 5: pH 8.59.

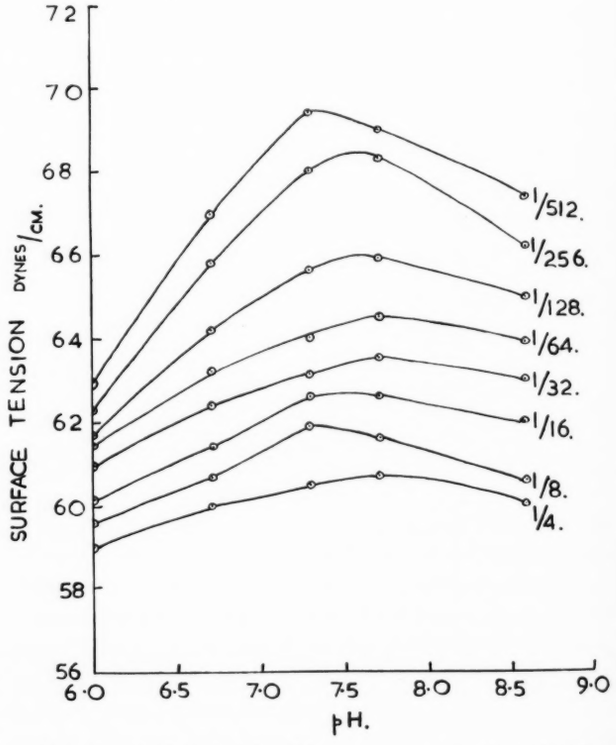

FIG. 4.—Surface tension of serum as a function of pH at various dilutions.

typical set of experiments on sample C12 which may be regarded as intermediate between typical cancer and noncancer sera. With borate and succinate buffers (6) the surface tension versus dilution curves are different from those obtained with acetamide solutions and approximate the curves obtained by diluting sera with water (Fig. 3). The

variation of the surface tension with pH exhibits maxima between 7.3 and 7.8 (12).

The surface tension versus dilution curves present a close analogy with the curves obtained for the system diluted serum-sodium cholate (22), which show a sharp surface tension minimum at 20–200 dilutions, approximately as in our experiments. This effect has been explained in terms of the combination of serum proteins with sodium cholate, which tends to displace lipids from the lipoproteins. The effect is more pronounced with sera from which the lipids have been removed. This, however, is difficult to apply to the MR, since γ_0 values of cancer sera are not higher than those of normal sera, in spite of the raised lipid content of the former; and the surface tension maximum observed with lipid-free sera by Tayeau and Blanquet and in our experiments is not explained. Conversely, Elkes and Finean in their work on the hemoglobin-sodium hexadecyl sulfate system noted the formation of soluble and insoluble complexes at significantly different, limited ratios of detergent to protein (8). Elkes and Finean suggested different mechanisms of combination on the acid and alkaline sides of the isoelectric point. In the case of the amphoteric acetamide an approximately symmetrical behavior is expected. The behavior of the chemically simpler system acetamide-diacetamide (7) confirms the suggestion that the characteristic lowering of the surface tension in our MR is due to a combination of acetamide with serum proteins, resulting in the attraction of protein molecules into the surface. According to modern views on the nature of acetamide (17, 18), the combination is assumed to take place through hydrogen bonding. The finer mechanism of the process—in particular, whether film penetration occurs (9)—cannot be decided from the existing evidence. The maximum of the curves may represent the opposite process, which is reversed when a certain stage of denaturation has been achieved. At dilutions beyond this point a cloudiness occurs. This is not due to the precipitation of globulins owing to dilution, since globulin fractions of cancer sera dissolved in saline to the protein concentration of the original serum give a MR with acetamide which is almost identical with the MR of the original serum. Finally, it should be noted that the MR with acetamide is closely related to modern technics of tensiometric titrations (16, 24).

SUMMARY

1. The surface tensions of cancer and noncancer sera were determined in progressive dilutions with 5 per cent acetamide.
2. The difference between the initial and minimum surface tension values was found to be positive in pregnancy and most of the cancer samples, whereas it was negative in most of the other samples.
3. A conventional unit, the critical area, was defined and found to be positive in pregnant and most of the cancerous samples, whereas it was negative or slightly positive only in other samples.
4. The modified MR is discussed from the point of view of the combination theory.

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Neoplasms in Rats Treated with Pituitary Growth Hormone

III. Reproductive Organs*

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This paper deals with the neoplastic and hyperplastic changes in the ovaries, uteri, and mammary glands of rats treated with growth hormone. Abnormalities of these organs occurred in 12 of 15 female rats following the administration of pituitary growth hormone for long periods. The neoplasms occurring in the lungs, lymphatic tissues, and adrenal glands of these rats have been reported in the preceding papers of this series (8, 9).

After reaching the plateau in their growth, female rats of the Long and Evans strain, 237–239 days old, were injected intraperitoneally with pituitary growth hormone daily,[1] 6 days a week, for a maximum period of 485 days. The initial daily dosage of growth hormone was 0.4 mg.; this was increased at intervals to a maximum of 3.0 mg. A similar group of adult females was injected parenterally with comparable amounts of albumin.

Serial sections of the ovaries were prepared. The uterine horns were fixed, and blocks of tissue from both horns were taken for microscopic examination. The mammary glands were examined both by spreads and by sections. The mammary spreads were prepared by stripping the mammary tissue from the skin and fixing in formol. The spreads were stained with alum carmine and cleared in methyl salicylate.

TABLE 1

REPRODUCTIVE ORGANS OF CONTROLS

Animal	Period of treatment (days)	Age at autopsy (days)	Ovary	Uterus	Mammary gland
B6530	378	615			
B6512	438	671		adenocarcinoma of endometrium	
B6400	484	722			
B6508*	483	720		endometrial polyp	fibro-adenoma
W6523	483	720			fibro-adenoma
GH6442	485	722			
GH6554	485	722			
GH6357	484	722			
G6460	485	722	cystadenoma		
BH6262*	485	722			
G6268	484	723			fibro-adenoma
B6291	484	722			
BH6292	484	722		endometrial polyp	
B6492	485	722		glandular cystic endometrial hyperplasia	
BH6294	485	723		endometrial polyp	

* Rats B6508 and BH6262 had macroscopically visible tumors of the pituitary gland.

RESULTS

OVARIES

Control rats.—The ovaries of most of the controls were not remarkable except for certain changes previously reported in rats of the same age. Follicles and corpora lutea in various stages of normal development and regression were pres-

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[1] The pituitary growth hormone used in this study was pure by physicochemical criteria (6).

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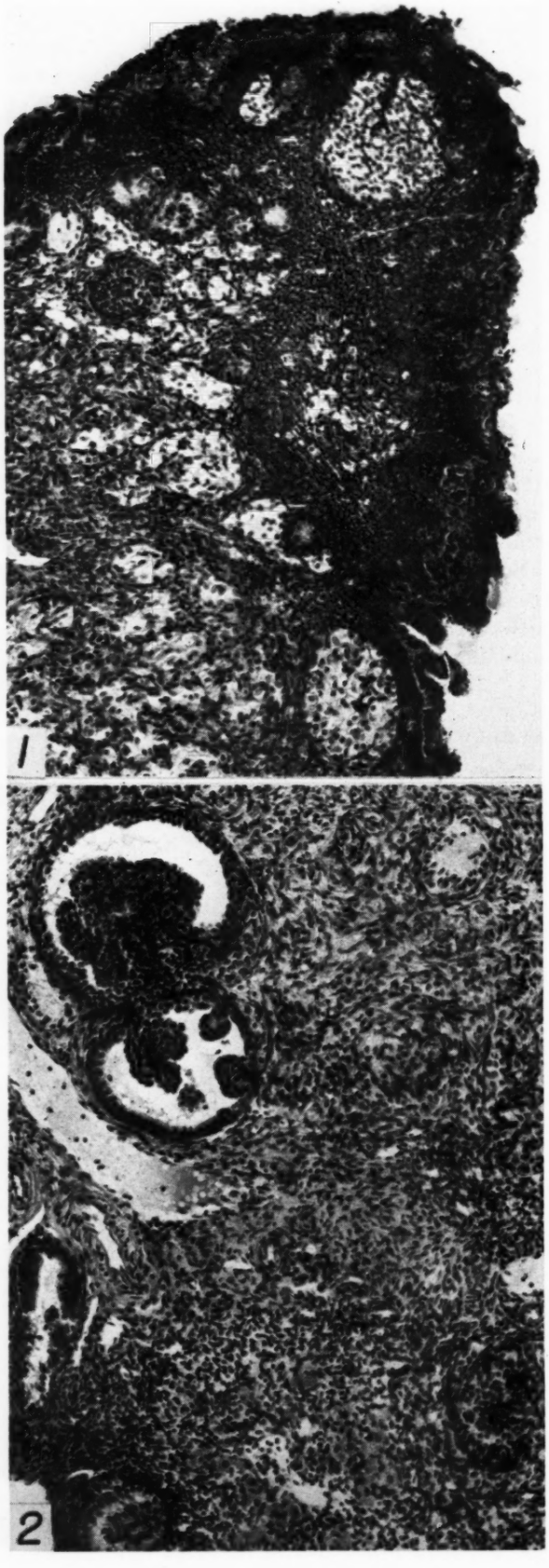


Figs. 1 and 2.—Control rats. Ovaries

ent in twelve rats (Table 1). In the remainder of the controls, cystic follicles and very old corpora lutea were present.

As reported previously concerning rats of comparable age, there were pseudo-testicular tubules in all ovaries (3). These structures were formed by pale cells resembling Sertoli cells. In nine rats there were many spherical structures composed of small cells with clear cytoplasm and small round nuclei with scanty coarse chromatin granules (Fig. 1). These cells were apparently derived from the theca interna of involuting follicles. Focal areas of papillary hyperplasia of the germinal epithelium were present in five rats. In one rat there was a small papillary cystadenoma composed of low columnar cells (Fig. 2).

Experimental rats.—The ovaries of the experimental rats were quite different from those of the controls (Table 2). Although follicles and corpora lutea were present in all but two animals, the follicles were not as numerous, and recently formed corpora lutea were only occasionally present. Papillary proliferation of the germinal epithelium occurred in eight rats. Small papillary cystadenomata were present in two rats. Pseudo-testicular tubules were more numerous and often larger than those noted in the controls. Bizarre, small, atypical follicles were present in six rats. Solid tumors of the ovaries were present in two rats.

In the eight rats with papillary proliferation of the capsule, the papillary processes were composed of pseudo-stratified columnar cells with elongated nuclei. These areas were very numerous in both ovaries of one rat (Fig. 3) and in another rat were associated with a cystadenoma (Fig. 4) of the same ovary and multiple tumors of the opposite ovary. The cystadenomata which were present in two rats were small and lined by columnar cells with basally placed hyperchromatic nuclei. The nuclei varied slightly in size and shape. Mitoses were rare.

Pseudo-testicular tubules similar to those observed in the ovaries of the control rats were also present in the ovaries of thirteen of the experimental rats. These tubules were usually larger and more numerous than those in the controls. Areas composed of large interstitial cells with abundant

Fig. 1.—Rat B6508. The germinal epithelium shows papillary hyperplasia. Several atretic follicles composed of abnormally pale thecal cells lie immediately beneath the germinal epithelium. The remainder of the tissue shows very numerous pseudo-testicular tubules and interstitial cells. This degree of abnormality was not encountered in any of the other controls. This animal had an adenoma of the pituitary gland. ×125.

Fig. 2.—Rat G6460. A small papillary cystadenoma is present. There are four tubular structures, two of which are of the pseudo-testicular type. ×125.

pale cytoplasm were present in five of the experimental rats; pseudo-testicular tubules were very numerous in these areas (Fig. 5).

The small atypical follicles were characterized by absence of ova and proliferation of the cells of the granulosa and theca interna. The granulosa cells had hyperchromatic, irregular nuclei. Occasional mitoses were present in both the granulosa and theca interna. The outlines of these follicles were often very irregular (Figs. 6, 7).

In one rat (W6324) the right ovary was grossly involved by neoplastic changes. The left ovary revealed proliferation of the capsule as stated above. The neoplastic right ovary weighed 336 mg., and in this ovary there were three separate tumors (Fig. 8). Two of the tumors showed a mixed pattern which suggested a granulosa-cell tumor in some areas and interstitial-cell tumor (luteoma) in other areas. In the areas with the pattern of a granulosa-cell tumor (Fig. 9) the cell types were very similar to those of the atypical involuting follicles described above. Cells resembling those of the granulosa occurred in small compact masses and rosettes; these masses and rosettes were separated by cells resembling those of the theca interna. In areas showing the pattern of a luteoma or interstitial-cell tumor, the cells were moderately large with abundant cytoplasm. In most of these areas the cells were closely packed together; in some areas the cells were quite large and had very pale or clear cytoplasm (Fig. 10). A cyst was present in association with one tumor. The third tumor was composed of numerous large pseudo-testicular tubules with interspersed large pale interstitial cells (Fig. 11). The pattern of this lesion was similar to that of a testicular adenoma of the ovary.

A small, solid tumor was present in rat BH6334. This tumor had the pattern of a granulosa-cell tumor. Small atypical follicles were present. The remainder of the ovary was composed of large pale interstitial cells and some pseudo-testicular tubules.

TABLE 2

REPRODUCTIVE ORGANS OF RATS INJECTED WITH GROWTH HORMONE

Animal	Period of treatment (days)	Age at autopsy (days)	Ovary	Uterus	Mammary gland
G6534	350	587	atypical small follicles	adenocarcinoma of endometrium	
B6458	378	615	cystadenomata		
GH6368	380	618			fibro-adenoma
BH6274	412	641			fibro-adenoma and fibromata
BH6334	430	668	solid tumor atypical small follicles	glandular cystic endometrial hyperplasia	fibro-adenoma
G6506	432	669	atypical small follicles		
BH6276	435	673			fibro-adenomata
G6553	465	702	atypical small follicles		
BH6313	484	722	atypical small follicles		fibro-adenomata
W6324	483	721	multiple tumors of rt. ovary, cystadenoma, left ovary		fibro-adenomata
G6255	485	724		glandular cystic endometrial hyperplasia, endometrial polyp	fibro-adenomata and fibromata
G6361	484	722			
B6266	484	723			
G6269	484	723			fibro-adenomata and fibromata
G6271	484	723	atypical small follicles		

UTERI

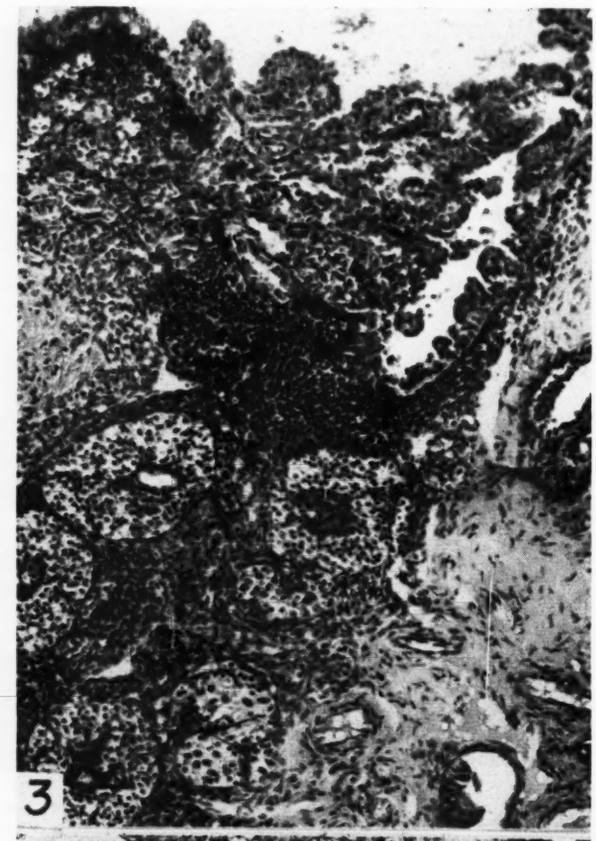
Control rats.—In ten rats the uteri were normal (Table 1). There were a few small endometrial glands. The epithelium was low columnar, and the stromal cells were small. Glandular cystic hyperplasia of the endometrium was present in one animal; endometrial polyps were found in three; and an early adenocarcinoma of the endometrium was present in one rat.

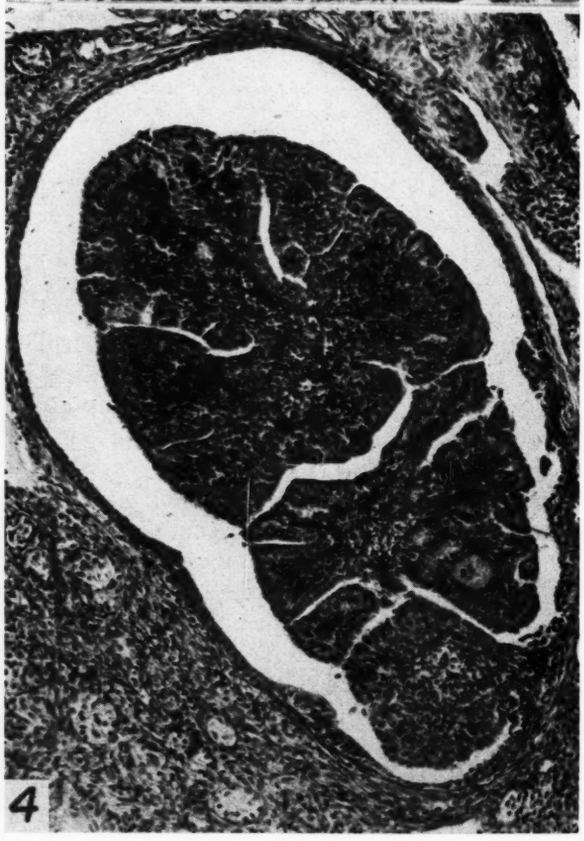
The glandular cystic endometrial hyperplasia was characterized by numerous large and frequently dilated endometrial glands composed of columnar cells. The stroma was edematous, and the cells were slightly enlarged.

The polyps were formed of large endometrial glands. The epithelial cells were columnar in most glands. There was cystic dilatation of some of the glands, and in these the epithelium was flat. The cells of the stroma were normal.

In one rat an adenocarcinoma of the uterus was found microscopically, although there was no grossly visible abnormality of the involved horn. The glands of the tumor were composed of large atypical epithelial cells with abundant cytoplasm and hyperchromatic nuclei. Occasional mitoses were present. The myometrium was not invaded.

Experimental rats.—There was hypertrophy of





FIGS. 3 and 4.—Experimental rats. Ovaries

the myometrium in all rats. The endometrium was normal in eleven rats. Glandular cystic hyperplasia of the endometrium was present in two rats, and in one of these there was also an endometrial polyp. A polyp of the endometrium was present in one additional rat. In one rat (G6534) the entire right uterine horn was greatly enlarged to a diameter of 4.5 cm. by a tumor, and it was adherent to loops of bowel. The neoplastic epithelial cells were

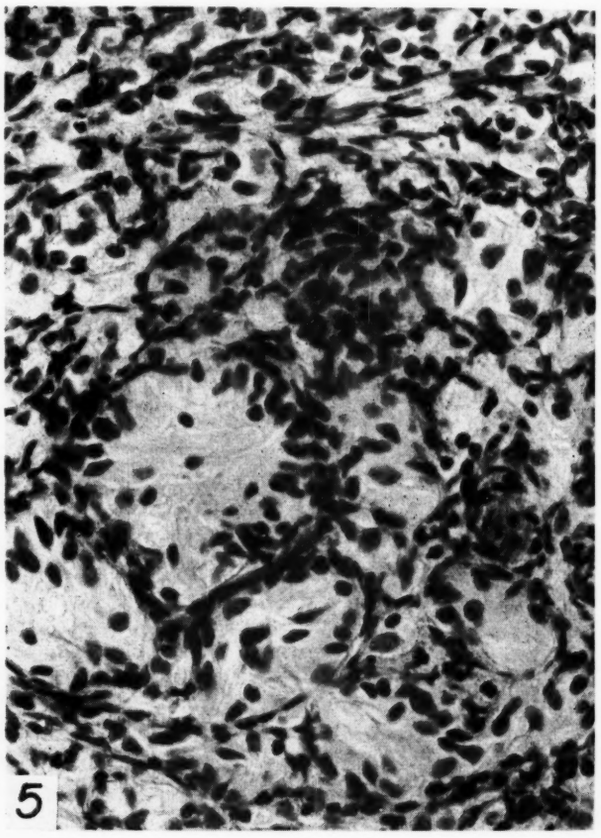


FIG. 5.—Rat G6255. Pseudo-testicular tubules and large pale interstitial cells. ×355.

large and had large, irregular, hyperchromatic nuclei. Mitoses were numerous. These cells were arranged in atypical glandular structures and small compact groups. The myometrium was extensively infiltrated and replaced by tumor. There were many areas of necrosis with polymorphonuclear leukocytic infiltration and abscess formation (Table 2).

FIG. 3.—Rat G6255. There is marked papillary hyperplasia of the germinal epithelium. The cortex contains many follicles undergoing atresia; the cells of the theca interna are abnormally pale. ×125.

FIG. 4.—Rat W6324. Left ovary. A papillary cystadenoma composed of columnar epithelial cells is present. The tissue at the lower left is composed of small pseudo-testicular tubules. (See Figs. 8–11, right ovary.) ×125.

MAMMARY GLANDS

Control rats.—In twelve rats the mammary tissue was of the virginal pattern; there were a few ducts, occasional alveolar buds, and rare, fully expanded alveoli. In two control rats there was lobulo-alveolar development[2] and solitary small fibro-adenomas; however, both of these rats (B6508 and BH6262) had adenomas of the pituitary gland. One additional control rat had a small fibro-adenoma. These tumors were characterized by proliferating interlobular and intralobular connective tissue which surrounded ducts and a few alveolar buds (Table 1).

Experimental rats.—There was lobulo-alveolar development varying from slight to marked in seven of the rats. The degree and distribution of the lobulo-alveolar development varied from animal to animal. There were areas showing only ducts and alveolar buds interspersed with areas of lobulo-alveolar development. In the rest of the experimental rats the mammary tissue resembled that of the controls and showed only ducts and alveolar buds.

Numerous fibro-adenomata of the mammary tissue were present in seven rats. Most of these tumors were larger than those occurring in the controls. In three rats with fibro-adenomata, the mammary tissue showed only ducts and alveolar buds; in four rats with fibro-adenomata, there was lobulo-alveolar development. In each of two rats (W6324 and G6269), there were eleven fibro-adenomata and fibromata. All the fibro-adenomata were similar and characterized by proliferation of the interlobular and intralobular connective tissue with no significant proliferation of the epithelium. Fibromata of the mammary tissue were present in three rats. The fibrous connective tissue forming these tumors was dense and sparsely cellular. There were no malignant tumors of the mammary tissue (Table 2).

DISCUSSION

The outstanding changes in the ovaries of rats injected with pituitary growth hormone were the presence of atypical small follicles and solid tumors. The absence of ova and the small size of the ovaries indicated that these abnormal follicles had

[2] The lobulo-alveolar development was marked in one animal (BH6262) and slight in the other (B6508).

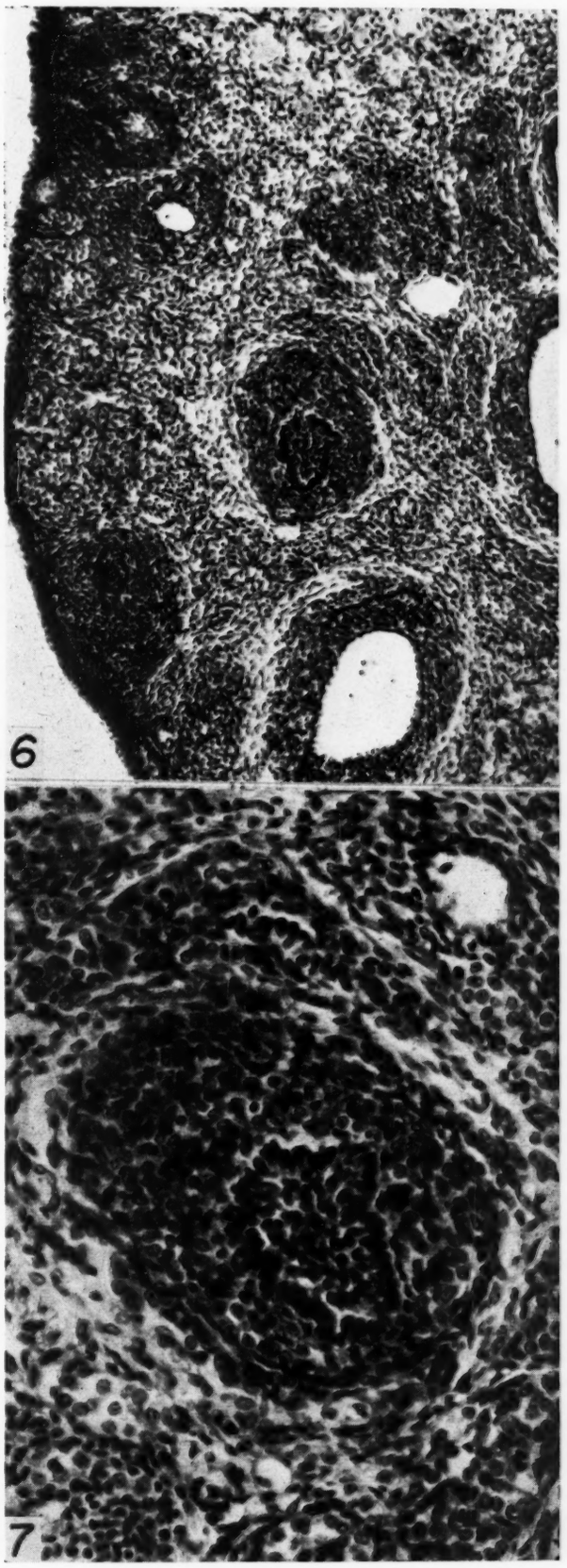


FIG. 6.—There are a number of small follicles. Some of these show normal atretic changes. Others show abnormal proliferation of the theca interna and granulosa. ×125.

FIG. 7.—Higher magnification of atypical involuting follicle. Note the marked overgrowth of the theca interna and irregular outlines of the proliferating granulosa. There are islands of normal interstitial cells. ×355.

FIGS. 6 and 7.—Experimental rat G6553. Ovary

undergone involutionary changes, whereas the mitotic activity and alterations in the general pattern of these follicles suggested abnormal stimulation. Our interpretation of these apparently dissimilar findings is that these changes are due to abnormal activity of the anterior pituitary superimposed on involuting follicles. It is considered likely that these atypical small follicles represent an early stage in the development of the solid tumors of the ovaries with the granulosa-cell and interstitial-cell tumor patterns. The occurrence of ovarian tumors in rats following the chronic administration of pituitary growth hormone was also noted in a previous similar experiment (3). Ovarian tumors have not been observed in hypophysectomized rats receiving growth hormone for similar periods (10).[3] It has also been shown that there are morphologic changes in the anterior pituitary following chronic administration of growth hormone to female rats after their weight reached a plateau. There was a decrease in acidophils and an increase in chromophobes; in two rats the histologic picture in the anterior pituitary was of the castration type (5). The above findings suggest that alteration in the function of the anterior pituitary gland following the administration of large amounts of growth hormone is an important factor in the production of ovarian tumors.

Of interest in connection with these ovarian tumors is the work of Biskind and Biskind (2), Li and Gardner (7), and Furth and Sobel (4). These investigators induced ovarian neoplasms by transplanting ovarian tissue into the spleens of castrate rats (2) and mice (4, 7). Biskind and Biskind observed that there was no growth of the intrasplenic transplants of ovarian tissue in rats that were hypophysectomized (1). It has been postulated (11) that this neoplastic transformation is dependent on inactivation by the liver of estrogens from the ovarian transplant in the spleen; this inactivation results in abnormal stimulation of the intrasplenic ovarian tissue by gonadotropins.

No remarkable differences were noted between the uteri of the experimental and control rats.

The mammary tissue in many of the experimental rats showed localized or generalized development; this was in noticeable contrast to the controls. In the latter group, excluding the two rats with spontaneous pituitary neoplasms, the mammary tissue consisted of a few ducts, occasional alveolar buds, and rare alveoli that were fully expanded. There were multiple fibro-adenomata of the mammary glands; these often reached considerable dimensions. A single small fibroadenoma was present in each of three control rats, and two of these rats had pituitary tumors.

SUMMARY AND CONCLUSIONS

1. The long-term administration of pituitary growth hormone to fifteen normal female rats after their weight had reached a plateau resulted in atypical hyperplasia of small follicles in five rats and in solid tumors of the ovaries in two rats. No similar changes were present in the control rats.

2. The atypical hyperplasia of small follicles is considered an early stage in the development of granulosa-cell and interstitial-cell tumors of the ovary.

3. Fibro-adenomata of the mammary tissue occurred more frequently, were larger, and were often multiple in rats injected with growth hormone.

4. The above changes are regarded as the result of the chronic administration of pituitary growth hormone which causes a marked disturbance in the normal function of the pituitary gland.

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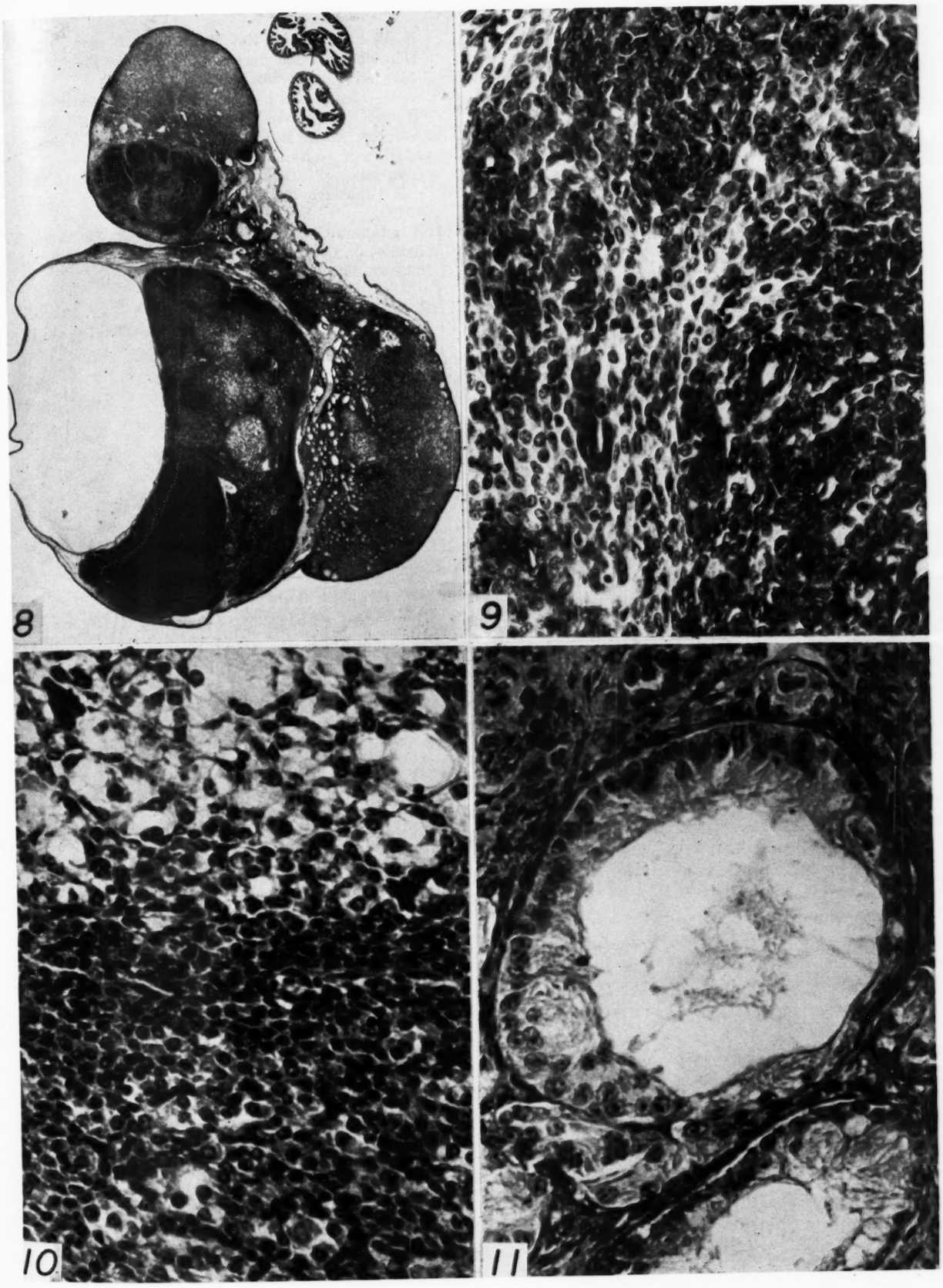
[3] These hypophysectomized rats were younger and received smaller amounts of growth hormone than the animals in the present report. Because of the greater sensitivity of these rats to growth hormone their rate of growth was comparable to that occurring in normal rats receiving much larger amounts of growth hormone. An additional group of hypophysectomized rats, of the same age as the rats in the present report, is being treated with growth hormone in amounts similar to that used in the present study.

Fig. 8.—Topographic view showing nodular masses of neoplastic tissue which have completely replaced normal ovarian tissue. ×8.

Fig. 9.—Higher magnification of one area showing pattern of granulosa-cell tumor. Note resemblance of the dark area to granulosa and the pale area to the theca interna of the atypical involuting follicle in Figs. 6 and 7. ×355.

Fig. 10.—This area has the appearance of an interstitial cell tumor. The cells in the lower half have a small to moderate amount of cytoplasm and are arranged compactly whereas in the upper half the cells are larger and form a reticulated pattern. ×355.

Fig. 11.—This was a separate tumor of the same ovary and it was composed entirely of pseudo-testicular tubules and interstitial cells. ×355.



FIGS. 8–11.—Experimental rat W6324. Right ovary

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The Histochemical Localization of Alkaline Phosphatase during Carcinogenesis in Rats Fed *p*-Dimethylaminoazobenzene*

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Greenstein in 1942 found a high content of alkaline phosphatase in transplanted rat hepatoma 31 (3). This tumor arose originally in the liver of a male Osborne-Mendel rat and was induced by the basal diet of White and Jackson (16) containing 0.06 per cent *p*-dimethylaminoazobenzene (DAB) with intermittent addition of 0.5 per cent cystine and 0.5 per cent methionine (17). The enzyme activity was determined with disodium phenylphosphate as substrate. Woodard in 1943 studied the alkaline phosphatase activity of livers of rats fed a diet containing 20 cc. of 3 per cent DAB mixed with 1,000 gm. brown rice and supplemented with carrots (19). Sodium β-glycerophosphate was used as substrate (20). It was noted that the average alkaline phosphatase activity, in units per gram of tissue, was 10 times higher in hepatic tumors than in normal liver. An intermediary stage following dye feeding, designated as precancerous, also showed a significant rise in alkaline phosphatase activity above normal liver, but not as high as that of hepatic tumors.

The histochemical demonstration of alkaline phosphatase activity has been reported by White, Dalton, and Edwards (17) for rat hepatoma 31 transplants and by Kabat and Furth (5) for primary hepatic carcinoma in the rat. The purpose of the present study was to ascertain where the increased phosphatase activity was localized in the liver of rats fed DAB and to follow the localization at intervals during the carcinogenic process. A preliminary report was presented in 1948 (14).

The taxonomy of liver tumors induced by DAB has been studied by several investigators, utilizing different basal diets (10, 11, 13, 17, 18). These tumors are complex histologically and are apparently influenced by diet. Further studies, such as those of Opie (10, 11), to determine the effect of diet variations on the histological appearance of the liver and tumor might be of great value in resolving some of the differences reported by various investigators.

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MATERIALS AND METHODS

Seventy-seven male Sherman rats were used in this study. They were placed on the following diet: casein ("Vitamin-free"), 120 gm.; glucose, 790 gm.; corn oil, 50 gm.; salt mixture (modified Phillips-Hart mixture), 40 gm.; and *p*-dimethylaminoazobenzene, 0.6 gm. This was supplemented, per kilogram of ration, by: thiamin chloride, 3.0 mg.; riboflavin, 2.0 mg.; pyridoxine hydrochloride, 2.5 mg.; calcium pantothenate, 7.0 mg.; choline chloride, 30.0 mg.; and cod liver oil, one drop/rat/month.

This diet has been worked out by Miner *et al.* (9) and by Miller *et al.* (8). Miller *et al.* (8) reported a tumor incidence of between 90 and 100 per cent, after 4 months on DAB.

Groups of animals were sacrificed after 1, 2, 3, and 4 months on the diet. The remaining animals were taken off the dye at 4 months and continued on the same diet without the dye for 1 month, at which time they were examined at autopsy. Sections from the liver were fixed in ice-cold acetone and processed according to the method of Gomori (2). Successive slides were incubated in sodium β-glycerophosphate at 37° C. for 5 minutes, 30 minutes, 12 hours, and 24 hours. Controls, in which the substrate was omitted, were run for each slide. No counter stain was used. Routine hematoxylin and eosin preparations were also made.

DESCRIPTION AND RESULTS

The 5- and 30-minute incubation periods serve to bring out the structures with intense phospha-

tase activity, while eliminating diffusion phenomena such as described by Martin and Jacoby (6). The slides incubated for 12 hours were chosen for the photographs, because they reveal all the cells on the slide. However, it is not possible to assert that structures staining only lightly after 12-hour incubations do so because of low but intrinsic enzyme activity, for their color may be due in part, or completely, to adsorption of materials from intensely colored areas. Apparent diffusion effects are considerably more pronounced after 24 hours of incubation.

The photographs show representative sections of: (*a*) livers of control animals (Figs. 1–3); (*b*) livers of animals on dye for 1, 2, 3, and 4 months (Figs. 4–8); (*c*) various portions of a complex liver tumor (Figs. 9–14); and (*d*) some special features of the tumors (Figs. 13 and 14).

Normal liver.—Essentially similar results were observed with fed animals and those on fast for 24 hours. Only the endothelium of the capillaries surrounding the bile ducts and an occasional mononuclear or polymorphonuclear leukocyte stained after 5–30 minutes of incubation. After 12 hours of incubation (Figs. 1–3), capillaries surrounding the bile ducts, the bile canaliculi, and the bile duct nuclei are intensely colored. In the parenchymal cells the nucleoli, chromatin, nuclear membrane, and cell boundaries stand out clearly. The cytoplasm exhibits a fine granular stippling. Often the cells nearer the portal areas are darker than those nearer the central vein (cf. Deane, [1]). To what extent diffusion from the portal area accounts for this picture is not clear. The same uncertainty holds for the more intense phosphatase activity of the hepatic cells, which is observed near the junction of bile canaliculi and bile ducts (Fig. 3).

Liver after 1–4 months of DAB feeding.—The most conspicuous alteration in livers of animals fed DAB is the marked increase in numbers of biliary and vascular epithelial cells.

In the sections incubated for 5 minutes only the infiltrating mononuclear and polymorphonuclear leukocytes stain darkly. The capillaries around the bile ducts show color, but the rest of the section is barely visible. In the 30-minute sections the vascular endothelium, as well as the leukocytes, is intensely stained. The nuclei of the biliary epithelium, and to a lesser extent those of the parenchyma, are slightly darkened.

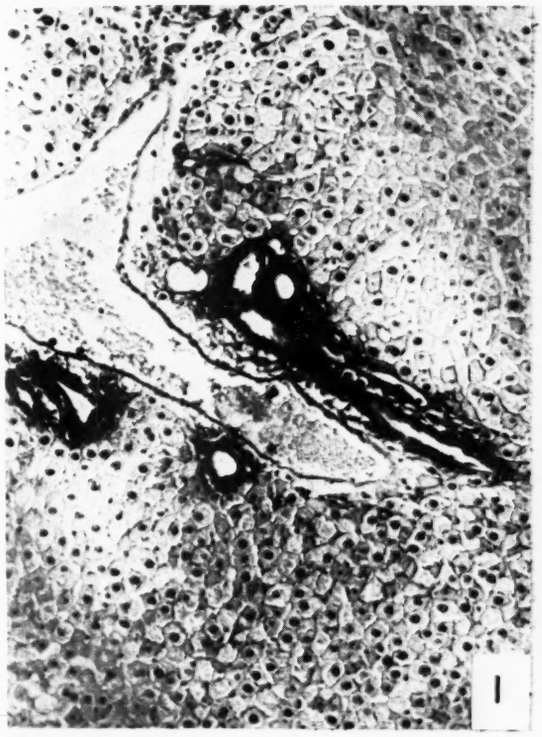
Figures 4–8 are of slides incubated for 12 hours, showing typical areas of biliary, vascular, and fibroblastic proliferation seen in livers of animals on dye for 1 month (Figs. 5 and 6), 2 months (Fig. 4), 3 months (Fig. 7), and 4 months (Fig. 8). Within these proliferative areas are visible many small ducts of varying size, with lumina of a few micra in the smallest ones, to almost 20 μ in the larger ones. When the bile ducts reach the size where the lumina are 15–20 μ wide (ca. 2 months), as much as half their external circumference is surrounded by closely approximated, newly formed vascular channels. These give an intense alkaline phosphatase reaction. As the ducts enlarge, these juxtapositional vessels become more organized and contain blood (2–3 months). In some instances, these vessels push the bile ducts into irregular channels, contributing to cystadenoma formation (3–4 months).

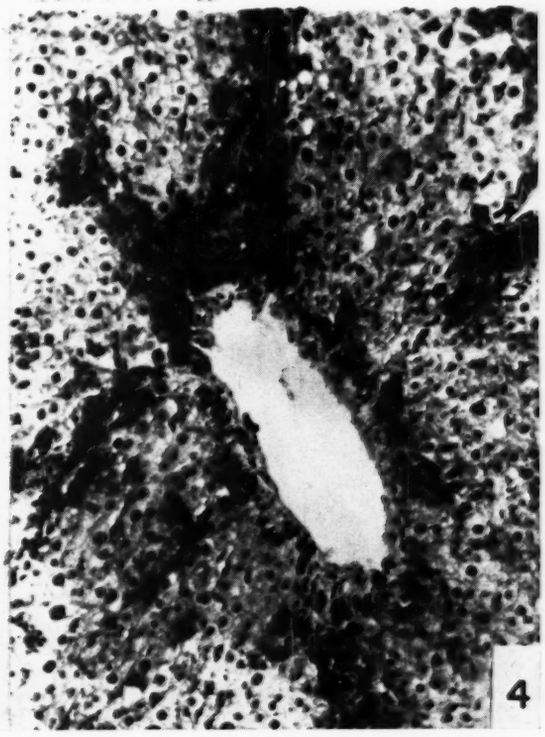
Everywhere around the bile ducts is epithelium, presumably biliary, composed of small cells indistinguishable from those of the ducts. Proliferation of fibroblasts also occurs; their nuclei show a less intense color than do the biliary cells. After 3 or 4 months of dye feeding these fibroblasts begin to organize into areas of cholangiofibrosis (Fig. 8). Not until the connective tissue becomes fairly dense do the collagen fibers show any significant phosphatase coloration.

Very early, the hepatic parenchyma cells give evidence of degenerative changes as a consequence of DAB feeding. This is particularly true in the region surrounding and ahead of the advancing column of newly formed bile ducts, but it is also, although less frequently, found in the cells surrounding the central veins. The cell boundaries give evidence of disintegration, and there is granular debris in the cytoplasm. The normal architecture of the liver lobules is lost as biliary epithelium, bile ducts, and new vessels invade them. By the end of the second or third month this invasion has extended to all parts of the lobule. Quite frequently, this isolates areas of parenchymal cells into "islands" (Fig. 8) (cf. Orr [13]). The cells are often distinctly larger than those of normal liver. These parenchymal cells give little or no evidence of alkaline phosphatase activity in the cytoplasm; the nuclei, and especially the nucleoli, are quite dark. The contrast between the light parenchymal islands and the intensely dark proliferative tissue surrounding them is striking.

Occasionally, within the parenchymatous tissue, there are foci of cells which give a more intense phosphatase reaction in both cytoplasm and nuclei.

Cholangiofibrosis becomes quite frequent by the end of the fourth month on the dye. The bile ducts, with lumina reaching 20–40 μ in diameter, are surrounded by connective tissue. The cells of the ducts show much less color than they did earlier in carcinogenesis. The most intense phosphatase reaction is given by the crescentic vascular





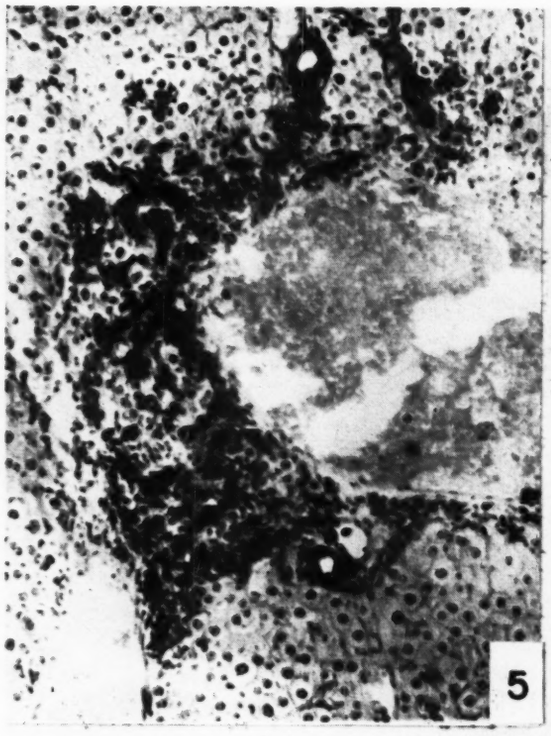
FIGS. 1–3.—Liver of normal fed rat

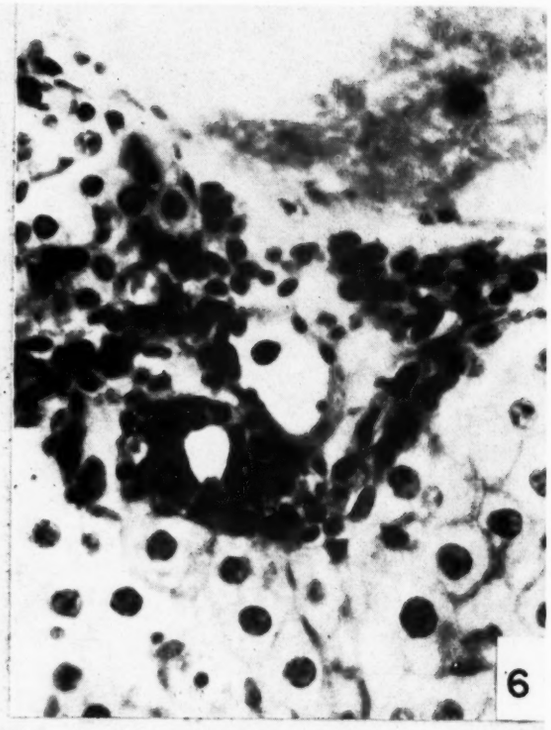
FIG. 1.—Low Power. Note intense color in capillaries surrounding bile ducts and in bile ducts. Mag. ×154.

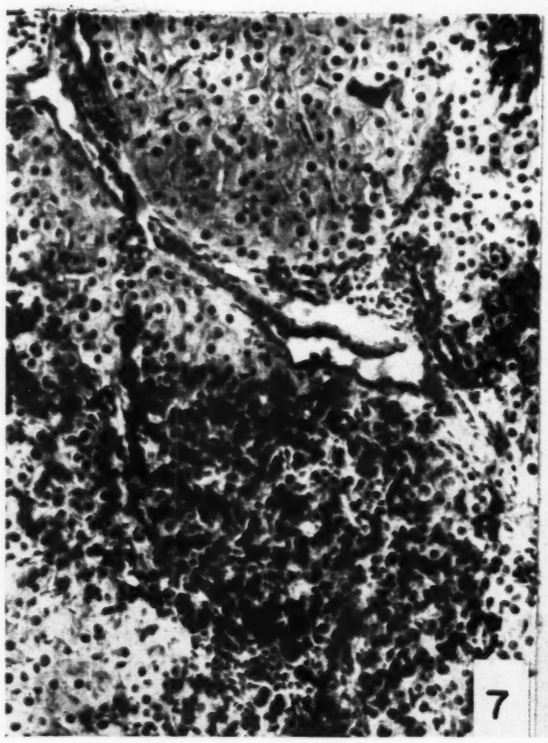
FIG. 2.—High Power. Note intense reaction of vessels surrounding bile duct. Mag. ×490.

FIG. 3.—Reaction of parenchymal nuclei and cell boundaries. In lower quadrant is a cross section of a small bile duct, probably at the junction with bile canaliculi; adjacent portions of parenchymal cells show more color. Mag. ×780.

FIG. 4.—Liver of rat #50 after 2 months on DAB. Note intense phosphatase reaction in region of proliferating biliary epithelium. Mag. ×154.







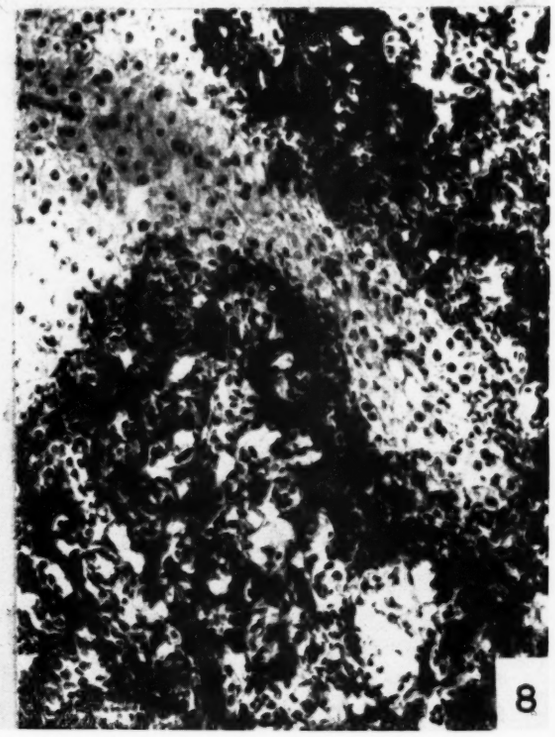


FIG. 5.—Liver of rat #44 after 1 month on DAB. Note marked proliferation of biliary epithelium, surrounding a dilated branch of the portal vein. Intense stain in polymorphonuclear leukocytes and biliary nuclei. Distinct lumen formations are visible. Mag. ×154.

FIG. 6.—Region of same area under higher magnification. Note intense phosphatase reaction in newly formed vessel adjacent to bile duct. Bile duct nuclei dark; fibroblast nuclei (elongated) lighter. Mag. ×490.

FIG. 7.—Liver of rat #51 after 3 months on DAB. Note small duct formations in area of biliary epithelium. Most intense color shown by vascular endothelium and infiltrating leukocytes. Mag. ×280.

FIG. 8.—Liver of rat #64 after 4 months on DAB. Note parenchymal "island." Above it, there is intensely stained biliary epithelium; below it, a beginning area of cholangiofibrosis. Mag. ×154.

endothelium surrounding the ducts. Many of the duct lumina are closed, apparently as a consequence of pressure from the outside and from intra-luminal proliferations of the duct itself. These intra-luminal proliferations show a more intense color than the duct wall from which they arise.

When cholangiofibrosis is present cystadenomata invariably occur. The pathogenesis of cystadenoma formation, as it appears in this material, is: (*a*) stasis by external compression by the fibrous and vascular stroma and (*b*), perhaps more important, excessive vascularization of the ducts with infoldings of epithelium.

Tumors.—Three of six animals killed at the end of 3 months of dye feeding showed the beginning of tumors composed of closely packed cells similar to the proliferating biliary cells of this and earlier stages.

Of the thirteen animals fed the dye for 4 months, nine showed the presence of well defined large tumors. Without an exhaustive study of serial sections of these tumors, an adequate description of their cell types is not possible. Nevertheless, it is of interest to record the results seen on some six or more sections of each of the nine tumors. Four tumors were composed exclusively of cells like those of the biliary epithelium, four contained areas of parenchyma-like cells in addition to areas of biliary cells, and one showed only parenchyma-like cells.

The complex and varied nature of the mixed tumors is demonstrated by Figures 9–14, all different regions of the same tumor, from slides incubated for 12 hours. The cells in Figures 10 and 11 are of biliary origin. In Figure 9 the biliary cells may be seen merging imperceptibly with cells which appear to be parenchymatous (and which would generally be referred to as hepatoma, in its strict sense).

Biliary carcinoma, adenocarcinoma, and cystadenocarcinoma are illustrated. The cells, while varying in detailed anatomic architecture, are considerably smaller than hepatic parenchymal cells and have a much larger nucleus-to-cytoplasm ratio. The cell membranes do not show up as distinctly as do those of parenchymal cells with this technic. But the cuticular border, in cells lining adenomata and cysts, shows clearly. In certain areas, the solid, unorganized masses of biliary epithelium show an intense color (Fig. 11), in striking contrast to the organized cells lining cavities. What little color the organized cells show is present in nuclear membrane and chromatin. Scattered among the masses of biliary epithelium, typical bile duct formations may be found.

In areas where there is a dense connective tissue stroma, the fibroblast nuclei and collagen fibers are darkly colored (Fig. 10). This is in contrast to the lack of color in the areas of early connective tissue. In many regions, the stroma takes the form of stalk-like formations containing many vessels, over which the tumor cells are arranged, generally stratified in several layers (Fig. 10). Where there is only one layer of cells, the cells resemble those of normal adult bile ducts.

The parenchymatous region ("hepatoma") of the tumor is composed of larger cells, with more abundant cytoplasm and more clearly visible cell membranes. They frequently show the large nucleoli characteristic of the normal liver parenchymal cells. There is little fibrous tissue among these cells, but there is a marked growth of small blood vessels, which show intense phosphatase activity (Fig. 9).

Adjacent to this "hepatoma" area is a typical cystadenoma formation (Fig. 12). Here, too, the cuticular border shows a positive reaction while the cell membranes between adjacent cells do not. The cells are usually arranged in a single column of cuboidal layers where the lumen is large, and stratified into two or three layers where the lumen is small. The interior of the stalk-like formations gives an intense phosphatase reaction, with the newly formed vascular spaces, biliary epithelium, and connective tissue darkly colored.

Figures 13 and 14 illustrate some other features of the tumors. In the bile duct tumors one frequently sees masses of cells within the lumen which can be traced to hyperplasia of the neoplastic bile ducts. These cells show intense color. Some appear to have swollen and dropped off into the lumen. Similar hyperplasias into the lumen of adenomata may occur in the diffuse "hepatomata." In the midst of the apparent "hepatoma" may be darkly staining small neoplastic ducts. These are similar to the non-neoplastic bile ducts seen in the liver after 1–3 months of DAB feeding.

In the sections of tumors incubated for 5 or 30 minutes, intense phosphatase activity is shown by: (*a*) the mononuclear and polymorphonuclear leukocytes which may be present, (*b*) the vascular endothelium, (*c*) the areas of dense connective tissue, and (*d*) areas of necrosis. The areas of unorganized biliary epithelium which stain very intensely after 12 hours of incubation are also considerably darker than the organized epithelium in the 5–30-minute preparations.

DISCUSSION

These studies demonstrate intense alkaline phosphatase activity in the regions of rapidly proliferating biliary epithelium which constitutes the

most impressive feature of the rat liver following the feeding of DAB. The activity seems to be especially high in the vascular sprouts which proliferate with the biliary epithelium and in the infiltrating white cells.

It is the progressive increase of this proliferative tissue which undoubtedly accounts for the increasing alkaline phosphatase activities which Robertson and Kretchmer (15) found by direct chemical assay of samples of the same livers we have described here. Similarly, it is these tissues which probably account for the high enzyme activity reported by Woodard (19) for "precancerous" livers.

On the basis of our findings, the level of alkaline phosphatase activity of a tumor appearing as a result of DAB feeding would be expected to vary with the extent of vascular and connective tissue stroma, infiltrated leukocytes, unorganized biliary epithelium, and necrosis. The more parenchyma-like cells that are present in the tumor, the more the phosphatase level would be expected to be like normal. It is of interest to note that Greenstein (3, 4) found that in the tumors of mouse liver, in which the tumor cells resemble normal liver parenchymal cells, and where biliary epithelium proliferation is not very prominent, there is no increased level of alkaline phosphatase activity. In the rat, Greenstein (3), Woodard (19), and Robertson and Kretchmer (15) found increased activity. The latter authors (personal communication) found the extent of this increase to be very variable from sample to sample.

At the completion of our studies, we became aware of a significant publication by Mellors and Sugiura (7). These authors report changes in the basophilia of liver cells following DAB feeding such as previously described by Opie (12). They found that in most instances the degree of basophilia was correlated with the intensity of alkaline phosphatase activity.

In our material, areas of regenerating parenchyma cells such as described by Opie and by Mellors and Sugiura are quite uncommon. Where they are seen, intense basophilia is not often present, if one may judge by the degree of hematoxylin staining. In the few instances where such basophilia was observed, intense color was also seen in the corresponding cells on the alkaline phosphatase preparations. To what extent this may be due to the high enzyme activity of adjacent bile canaliculi is not clear. A closer study of the basophilia-alkaline phosphatase activity relationship in our material is in progress.

It is difficult to judge what role, if any, increased alkaline phosphatase plays in tumor formation following DAB feeding. It is highly concentrated in the areas of non-neoplastic proliferating cells early in carcinogenesis. In the final tumors, it is highly concentrated only in the ancillary stroma and in the unorganized biliary epithelium. "Hepatomas" and the organized epithelium of biliary adenomatous formations are low in activity.

CONCLUSIONS

1. The increased alkaline phosphatase activity which occurs during the process of carcinogenesis in the liver of rats fed DAB is localized in the areas of rapidly proliferating biliary epithelium; vascular sprouts and infiltrating leukocytes show most intense activity.

2. In the tumors produced after 4 months of DAB feeding, the enzyme activity is localized in the ancillary stroma, infiltrating leukocytes, necrotic tissue, and areas of unorganized biliary epithelium. Both the organized epithelium of biliary adenomatous formations and "hepatomas" are low in activity.

ACKNOWLEDGMENTS

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FIG. 9.—Tumor of rat #74 after 4 months on DAB and 1 month on dye-free diet. Shows diffuse "hepatoma" composed of parenchyma-like cells. Note light staining in contrast to intense color of vascular epithelium. Barely visible in the left half of the photograph are duct formations in small islands of biliary epithelium. Mag. ×154.

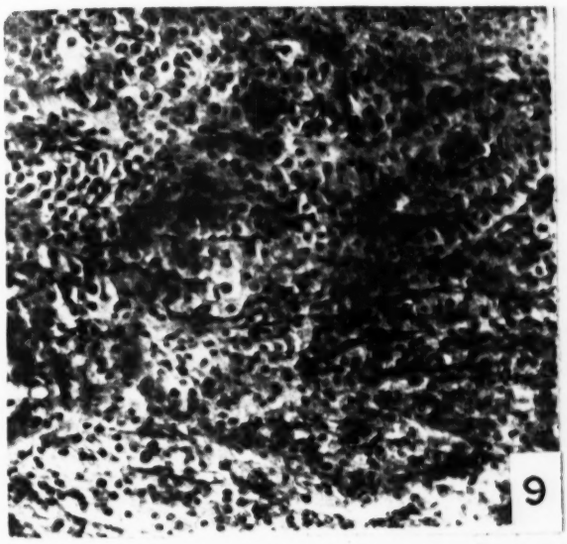
FIG. 10.—Another area of same tumor. Note: (*a*) strong enzyme reaction in connective tissue stalks supporting the tumor and (*b*) the lightly-colored nuclei of the tumor cells. Mag. ×154.

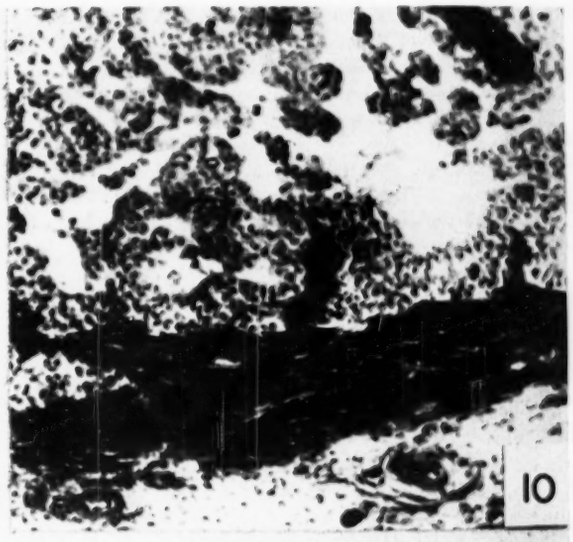
FIG. 11.—Another area of same tumor. Note: (*a*) the dark color of the unorganized biliary epithelium cells in the trabecuia and (*b*) the light color of the cells organized into adenomata. Mag. ×154.

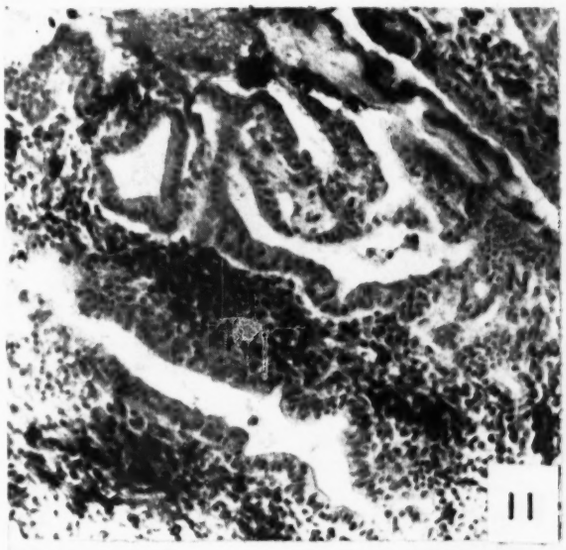
FIG. 12.—Another area of same tumor. Shows cystadenoma formation. Intense color is seen in stalk composed of connective tissue, blood vessels, and biliary epithelium. Mag. ×154.

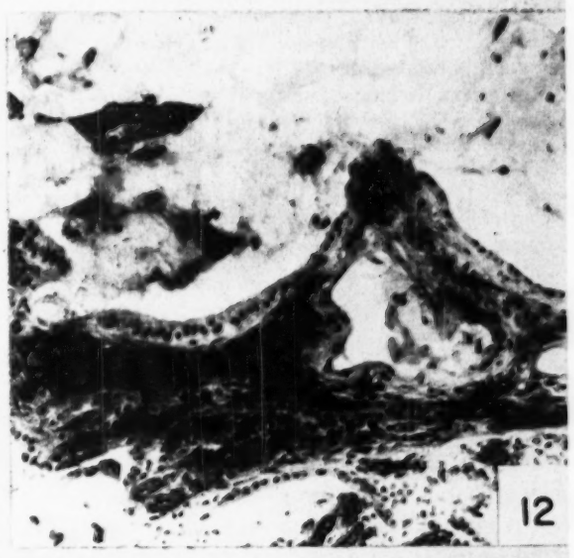
FIG. 13.—Area of same tumor. Shows adenocarcinoma. Note hyperplasia of inner lining of neoplastic bile duct. Mag. ×490.

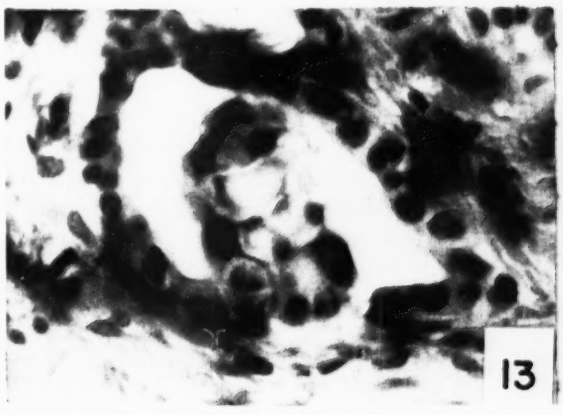
FIG. 14.—Another area of same tumor. Note lightly-stained cells of large bile duct in upper left corner. Immediately below this duct are dark cells of small duct. Note the darkly-staining bile duct cut longitudinally, and immediately above it, an aberrant liver cord formation. Mag. ×490.

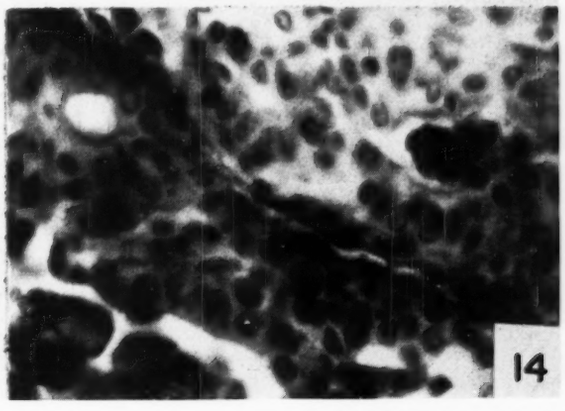












FIGS. 9–14

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Effect of Sulfonated Azo Dyes on Mouse Tumors

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The possible relationship between deficient reticulo-endothelial function and neoplasia has been investigated by several workers (7). Some investigators utilized the hypothetical relationship between reticulo-endothelial function and tumors for therapeutic approaches; in other words, attempts were made to combat malignant tumors by methods which were expected to increase reticulo-endothelial activity. These mostly unsuccessful endeavors will not be reviewed here; however, it may be remarked that in most instances complex biologic stimuli were employed for the purpose of enhancing reticulo-endothelial function. It was thought possible that stimulation of reticulo-endothelial tissues by means of chemical compounds, defined as to their structure and amenable to exact dosage, might represent a more promising approach (4).

The present study was prompted by a report made by Cestari (1) in 1940 regarding the effect of sulfonated Sudan IV (scarlet red) on the Kupffer cells in the rabbit. According to this author, prolonged intravenous injections of Sudan IV were followed by deposition of the dye in the Kupffer cells; from there it gradually passed into parenchymal cells, causing degenerative changes and proliferation of connective tissue, and this ultimately led to a condition resembling cirrhosis. When the author, however, used a sulfonated derivative of Sudan IV, which was more readily water-soluble, this dye was taken up by the Kupffer cells, caused them to proliferate in response to the storage, and was eliminated without any apparent damage to the liver.

On the basis of this report, it was thought worth-while to investigate the effect of sulfonated azo dyes on reticulo-endothelial activity and tumors of mice. A number of such compounds were made available by the National Aniline Division, Allied Chemical and Dye Corporation.[1] The compounds which have been tested so far are listed in Figure 1. Sudan IV (first horizontal column) was not used in our work but is included in the chart in order to show its structural relationship to the other compounds. All the dyes were readily water-soluble up to concentrations of 1–2 per cent. Subcutaneous injections of the dyes, dissolved in physiologic solution of sodium chloride to the desired concentration and autoclaved before use, were given to the experimental animals.

The first compound tested was cloth red B (CRB). Subcutaneous injections of 0.5 per cent solutions of the dye were given to groups of C57 black and C3H animals for periods ranging from 3 to 20 months. No storage of the dye in reticulo-endothelial cells could be observed. As was briefly reported on a previous occasion (6), moderate proliferation of Kupffer cells in the livers of animals, injected for a period of at least 6 months, could be demonstrated by injecting carmine solution intraperitoneally before sacrificing the animals and by counting the number of storing cells (5). C57 black mice responded to the dye with a more marked proliferation of Kupffer cells than did C3H animals. All the treated female animals of the C3H strain developed spontaneous mammary carcinoma. Likewise, tumor development in C57 blacks injected subcutaneously with methylcholanthrene (0.6 mg.) was not affected by twice-weekly injections of the dye.

In 1 out of 28 C3H mice, and in 1 out of 34 C57 black mice, none of whom had received carcinogen but which had been injected twice weekly with CRB, fibrosarcomas arose at the site of the injection of the dye; this occurred in the C3H animal 15 months and in the C57 black mouse 16 months after the start of the injections. Transplants of the tumors were carried in the respective strains for 21 and 6 passages, respectively. This might indicate a weakly carcinogenic effect of the dye; reference is made to the action of scarlet red itself, which, according to the data summarized by Hartwell (2), has shown carcinogenic activity in isolated instances. More recently, Smith (3) demonstrated its co-carcinogenic properties.

Only brief mention need be made of the results obtained with the compounds wool red 40F, solan-

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tine 8BLN, and brilliant scarlet red 3R. None of them exhibited any influence on the development of growth rate of induced or transplanted sarcomas in C57 black mice. Of these dyes, only brilliant scarlet 3R showed slight storage in reticulo-endothelial cells.

The following data were obtained with Erie Fast Rubine B Conc. (EFR). This compound was readily stored in reticulo-endothelial cells, especially in the liver and spleen. The minimal lethal dose of the dye was approximately 0.6 mg/gm. The dosage actually used was 0.5 cc. of a 0.25 per cent solution for animals averaging 20–25 gm., or approximately 0.06 mg/gm (one-tenth of the

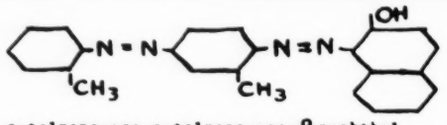
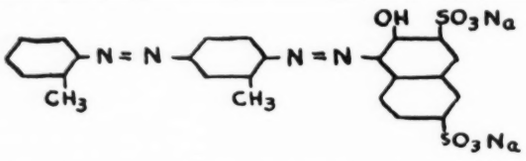
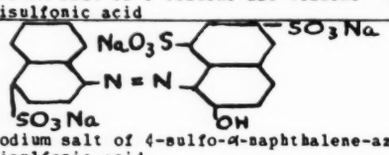
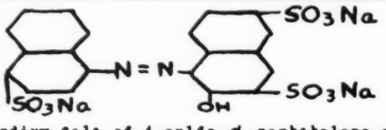
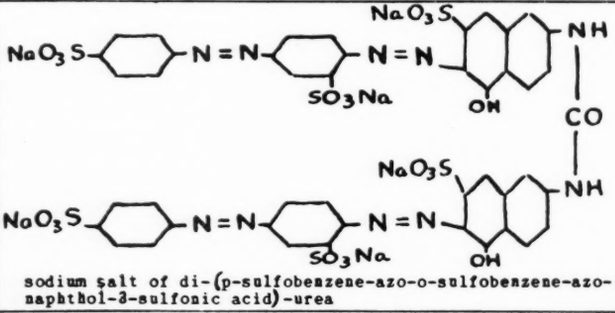
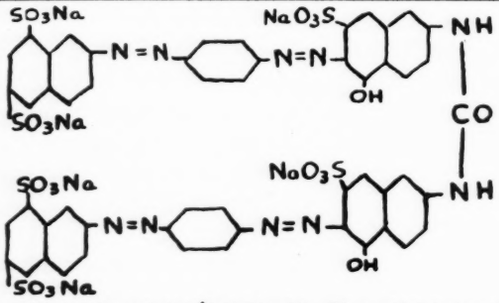
COMPOUND	COLOR INDEX	STRUCTURE	NUMBER OF SULFO GROUPS
SUDAN IV	258	 o-toluene-azo-o-toluene-azo-β-naphthol	0
CLOTH RED B	262	 sodium salt of o-toluene-azo-toluene- β-naphthol-8, 6-disulfonic acid	2
BRILLIANT SCARLET 3R PURIFIED	—	 sodium salt of 4-sulfo-α-naphthalene-azo-β-naphthol-6, 8-disulfoaic acid	3
WOOL RED 40F	184	 sodium salt of 4-sulfo-α-naphthalene-azo-β-naphthol-3, 6-disulfonic acid	3
SOLANTINE RED 8 BLN	—	 sodium salt of di-(p-sulfobenzene-azo-o-sulfobenzene-azo-α-naphthol-3-sulfonic acid)-urea	6
ERIE FAST RUBINE B Conc.	—	 sodium salt of di-(6, 8-disulfo-β-naphthalene-azobenzene-azo-α-naphthol-3-sulfonic acid)-urea	6

FIG. 1.—Sulfonated azo dyes tested in this study

minimal lethal dose). With a few exceptions, animals tolerated this twice-weekly dosage well and did not lose weight. In some animals ulcerations of the skin, which were probably the result of inadvertent intracutaneous administration of dye solution, occurred after prolonged periods of injections. If such ulcerations occurred near tumors, or near the site of the tumor inoculum or the deposit of carcinogen, the animals were excluded from further experimental observation.

per cent methylcholanthrene in olive oil (0.6 mg.). Group II continued to receive the dye injections twice weekly until the end of the experiment. The dye was injected at the farthest possible distance from the carcinogen deposit.

Figure 2 illustrates the delayed development of tumors in the experimental animals, as compared with the controls. Weekly examination included weighing of the animals, searching for tumors, and measuring of tumors present. The weight of the

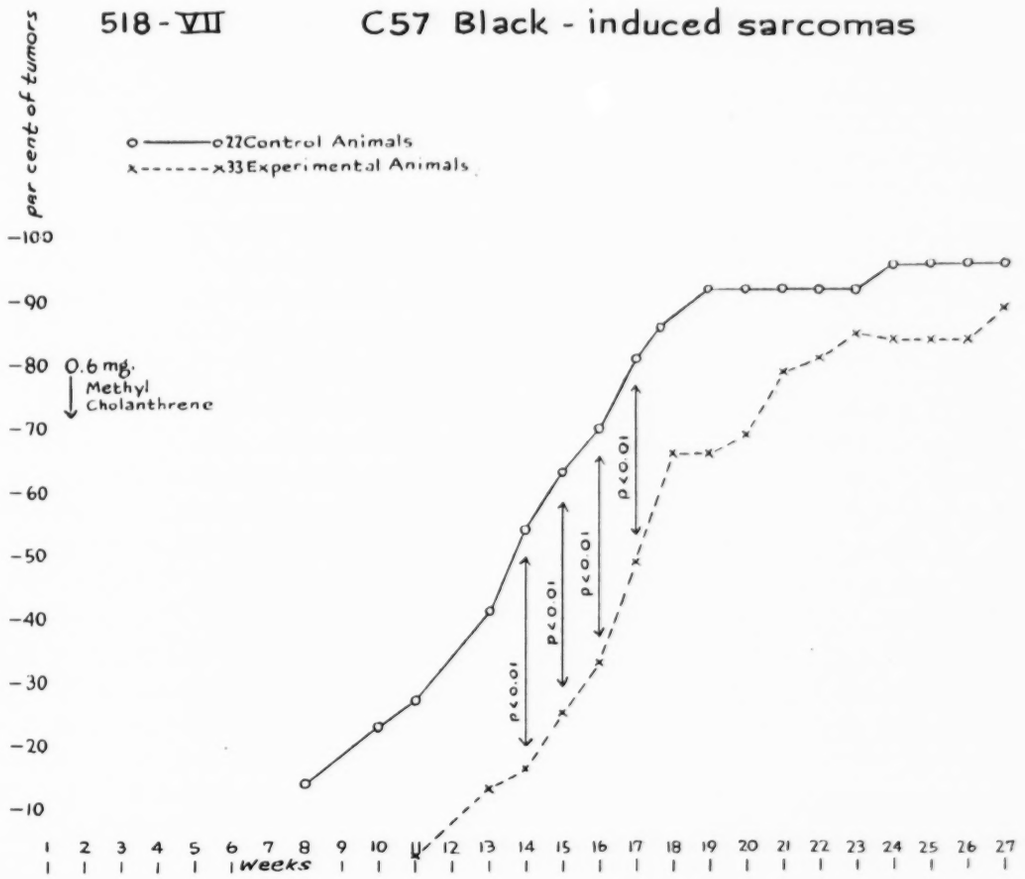


FIG. 2.—Effect of EFR on the development of methylcholanthrene-induced sarcomas in C57 black mice

The administration of EFR resulted in an inhibition of two types of mouse tumors, namely, a transplanted mammary carcinoma in C3H animals and a sarcoma induced by methylcholanthrene in C57 blacks. Representative experiments of these series are detailed.

Experiment 518-VII.—For this experiment, 5-month-old C57 black males were used. Group I consisted of 22 controls; Group II of 33 experimental animals. Group II received six subcutaneous injections of 0.5 cc. of 0.25 per cent EFR, given twice weekly during 3 weeks. Three days later, animals of both groups were injected subcutaneously near the scapula with 0.3 cc. of 0.2

injected animals did not vary from that of the controls by more than 5 per cent—the higher weight of the controls being due to the presence of larger tumors. This was corroborated by establishing the true weights of the animals at death or at the end of the experiment; the average weight of the controls was 17.4 gm., and that of the injected animals was 18.4 gm. A significant difference in the development of tumors was noted up to 17 weeks after administration of the carcinogen; during this period, calculation of p for the difference in the percentage of tumors gave values of less than 0.01, which are considered statistically significant. However, after this period the injected

animals started to develop new tumors, so that the difference between them and the controls became increasingly smaller. No difference in the growth rate of the tumors in, or in the survival time of, injected or control animals was noted.

Experiment 518-IX.—Four-month-old C3H males were used. Group I consisted of 15 controls; Group II of 15 experimental animals. Group II received a total of seven subcutaneous injections

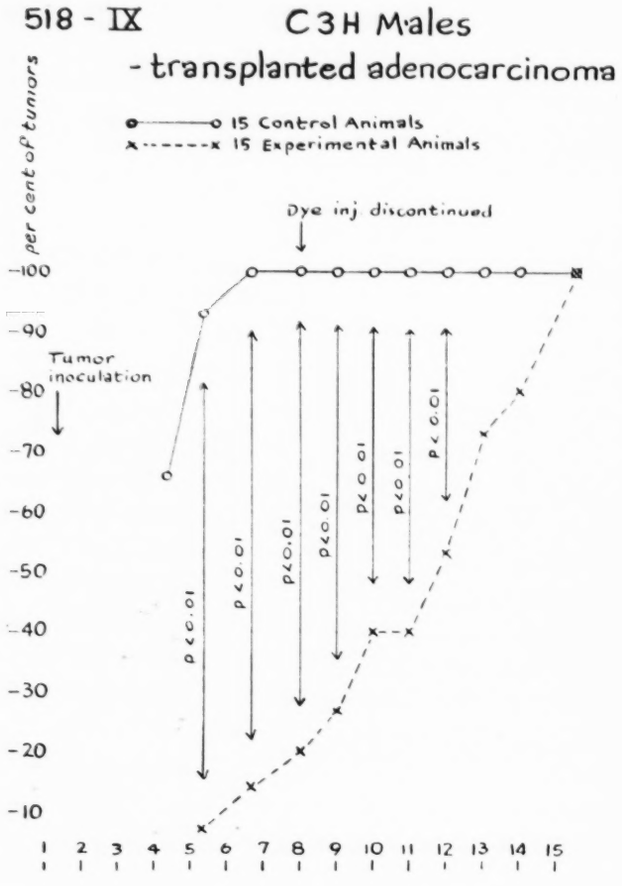


FIG. 3.—Effect of EFR on the development of a transplanted adenocarcinoma in C3H male mice.

of 0.5 cc. of 0.25 per cent EFR during a period of 3 weeks. Two days later, animals of both groups were inoculated with a transplantable mammary carcinoma. The dye injections were continued for 2 months after the inoculation. In Figure 3 the tumor development in both groups is recorded. Again there was a marked difference in the time of the appearance of the tumors in the two groups. This difference remained significant for a period of 11 weeks after inoculation, and after this time, just as in the previous experiment, the injected animals caught up with the controls, so that at the end the "takes" reached 100 per cent in both groups.

In addition to these experiments, others were performed with variations in the dosage and number of dye injections and in the timing of the injections, in relation to the administration of carcinogen or to inoculation of the tumor. These experiments showed either similar, or lesser, degrees of tumor inhibition. In none of the experiments was there a complete suppression of the tumor process in injected animals. Negative results were obtained in female mice of the C3H strain, in which injection of the dye was started at the age of 7 months; the incidence of spontaneous mammary carcinoma in this group reached almost 100 per cent, and the time of appearance and growth rate of the tumors was similar in injected and control animals.

Only transient, and statistically not significant, inhibitions of transplantable sarcomas in C57 blacks and transplantable melanomas and carcinomas in dba animals were observed.

In all experiments, similar gross and histologic findings were noted. Liver, spleen, and subcutaneous tissue showed, grossly, reddish-purple staining with the color intensity generally increasing with the length of dye administration. Tumors, when present, did not show staining except for a faint tinge in the adherent connective tissue.

Histologically, the most intensive storage of dye was found in the liver, which contained large numbers of dye-laden macrophages (Kupffer cells). Many of them were distorted or swollen, with displaced pyknotic nuclei (Figs. 4, 5). Occasionally, dye was found in accumulations of histiocytes in the vicinity of larger vessels. In some animals, the parenchymatous liver cells showed hardly any changes; in others, degenerative changes appeared with a moderate increase of binucleated cells. In later stages focal areas of liver

FIG. 4.—Liver of C57 black mouse. Storage of Erie Fast Rubine in Kupffer cells. No change apparent in hepatic parenchyma. Photomicrograph, ×150.

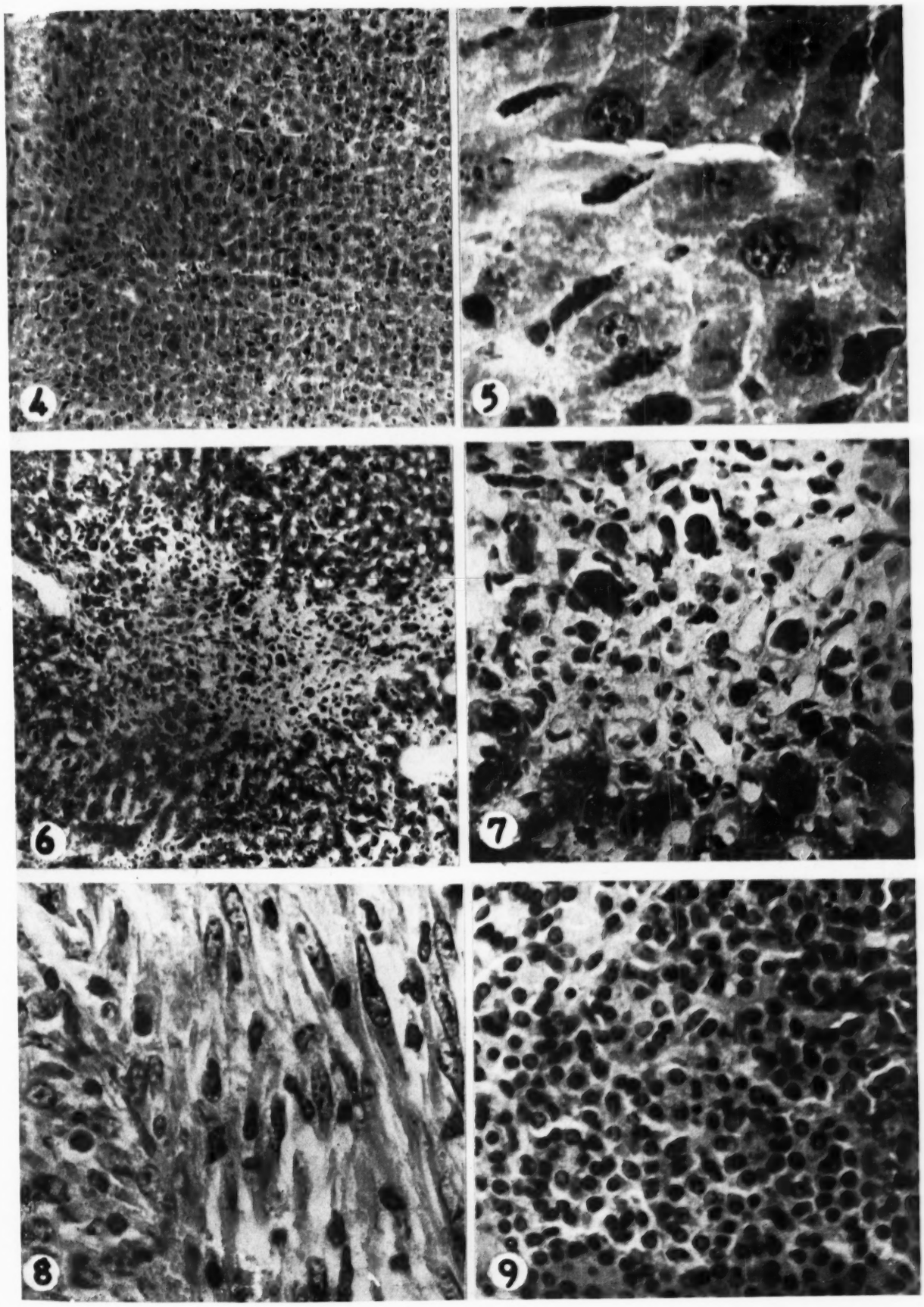
FIG. 5.—Higher magnification of Figure 4 showing macrophages laden with dye. Photomicrograph, ×500.

FIG. 6.—Liver of C57 black mouse injected with dye for 5 months. Note disorganization of hepatic structure and proliferation of connective tissue in central portion of field. Photomicrograph, ×150.

FIG. 7.—Higher magnification of central portion of Figure 6 discloses presence of dye-storing macrophages in the proliferating connective tissue. Note degenerated liver cells in lower margin. Photomicrograph, ×375.

FIG. 8.—Fibrosarcoma, induced by methylcholanthrene, in C57 black mouse injected with dye. Photomicrograph, ×375.

FIG. 9.—Transplanted adenocarcinoma, growing in C3H mouse injected with dye. Histologic features of this tumor and of tumor in Figure 8 were indistinguishable from those in tumors of control animals. Photomicrograph, ×375.



FIGS. 4–9

necrosis were noted, with dye accumulating in these areas. In some mice which had been injected for many months, severe alterations in the liver structure occurred, with large foci of proliferating histiocytes separating distorted liver cords (Figs. 6, 7).

It is noteworthy that, even 3 months after cessation of injections, dye was found stored in the liver and spleen. The latter organ presented variable numbers of storing macrophages within the red pulp, and dye was also found in the endothelial lining of sinusoids. The follicles contained no dye and appeared as white spaces in unstained sections; in some instances the peripheral follicular portion was replaced in hematoxylin-eosin-stained sections by homogeneous pink staining material, which, in unstained sections, contained dye in diffuse form.

In accord with the gross findings, neither carcinomas nor sarcomas showed dye storage in the tumor proper; dye-laden histiocytes were found in the peritumoral connective tissue. In none of the experiments, even when inhibition of tumor growth had been previously noted, were there any structural or cytologic differences between tumors of injected animals and those of controls (Figs. 8, 9).

After prolonged dye administration, delicate dye granules were also found in the tubular epithelium of the kidneys. No urinary excretion of the dye was noted at any time. After dye administration had continued for many weeks, dye was found in the blood serum of the mice.

DISCUSSION

The above experiments were carried out for the purpose of finding out whether it is possible to increase the resistance of mice to neoplasia by means of administration of dyes which are stored in reticulo-endothelial tissue. A moderate transient inhibition of certain types of mouse tumors was observed with the sulfonated azo dye Erie Fast Rubine Conc. B.

In connection with these findings the following points may be considered. Significant inhibition of tumors was found only in two types of tumors: (*a*) sarcoma induced by methylcholanthrene and (*b*) a transplanted mammary carcinoma, both of which showed a rather long latent period of induction or of "taking," respectively. This might indicate that resistance brought about by the dye is operative only in the presence of slow neoplastic processes.

The absence of histologic changes in tumors of treated animals is evidence against a direct action of the dye on tumor cells; it is possible that it acts indirectly by way of increasing some defensive mechanisms in the host, which we hypothetically connect with the reticulo-endothelial proliferation in the treated animals.

While the results of the present work have failed to demonstrate any remarkable effects of the administration of sulfonated azo dyes, they might have some value since they show an instance in which proliferation of reticulo-endothelial tissue was accompanied by a delay in carcinogenesis.

SUMMARY

Erie Fast Rubine Conc. B., one out of five sulfonated azo dyes tested, was found to be readily stored in reticulo-endothelial cells of mice. Injections of this dye into mice caused a temporary inhibition of the development of sarcoma induced by methylcholanthrene in C57 blacks and of a transplanted mammary carcinoma in C3H mice. An interpretation of these findings was presented.

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Further Studies on Natural Antisheep Agglutinins in Mice of Inbred Strains*†

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INTRODUCTION

In earlier work, natural and immune hemoantibodies were studied in mice of inbred strains with low and high spontaneous tumor incidence (5, 6). A difference between different strains was observed in that natural antisheep agglutinins occurred commonly in C57 black mice, whereas these antibodies were found much less frequently in five other strains (C3H, dba, Marsh-albino, B alb C, Akm); as a rule their titers were, when present, considerably lower in these strains. A number of questions arose in connection with this difference in hemoantibodies by which the C57 black strain is apparently set apart from other inbred strains. The present report deals with attempts to find answers to some of these questions, particularly with regard to the origin and nature of the agglutinins.

EXPERIMENTAL

The technics used were identical with those reported previously (6).

Antigenic structure of mouse tissues and antisheep agglutinins.—We have previously considered the possibility that the presence or absence of natural antisheep agglutinins, as found in the different mouse strains, may be the expression of antigenic differences existing in these strains (6). That is, the situation might be similar to the relationship between isoagglutinins and isoagglutinogens in man: the presence of the antigen excludes the presence of the homologous antibody. Antigenic differences between inbred mouse strains were demonstrated by Gorer (11, 12), who reported the finding of certain antigens in red blood cells in some, and not in other strains. Recent work by Maculla (16) was concerned with antigenic analysis of mouse tissues, but without consideration of possible strain differences.

We attempted to answer the following questions: Does the absence of natural antisheep agglutinins depend on the presence of the homologous antigen in red blood cells or tissues, and vice versa? Or are there merely quantitative differences of the same type?

In order to test this assumption, pooled serum of C57 blacks containing natural antisheep agglutinins was absorbed with tissues and red cells of mice of different strains. Tissues of C3H and dba animals were taken from animals without antisheep agglutinins in their serum, while tissues of C57 blacks were derived from animals with antisheep agglutinin titers of at least 1:8. Absorptions of the serum with fresh and boiled sheep cells, guinea-pig kidney, and kaolin suspensions served as controls. Two parts of 50 per cent suspensions of the antigens were mixed with 1 part of serum. The mixtures were incubated, with repeated shaking, for 1 hour at room temperature, and then the supernatant was separated for testing. The results of one such experiment are summarized in Table 1.

TABLE 1
RESULTS OF ABSORPTION OF POOLED C57 BLACK SERUM

	Titer
Unabsorbed	16
After absorption with:	
Fresh sheep cells	0
Boiled sheep cells	16
Guinea-pig kidney	8
Kaolin	16
Kidney, C3H	16
Kidney, dba	16
Kidney, C57 black	16
Spleen, C3H	8
Spleen, dba	16
Spleen, C57 black	16

With the exception of fresh sheep cells, none of the antigens removed significant amounts of the antisheep agglutinins, and, in particular, no differences

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were noted in the absorbing ability of mouse tissues derived from different strains. Identical results were obtained in absorptions with liver and erythrocyte suspensions of the three strains. The failure of guinea-pig kidney and of boiled sheep cells to remove the antibodies confirms our earlier findings (5) that the natural antisheep agglutinins in mice are not heterophilic antibodies of the Forssman type.

Further studies of the antigenic properties of mouse tissues included investigation of their ability (*a*) to produce antibodies in suitable experimental animals and (*b*) to react *in vitro* with the antibodies so produced.

Several years ago Brown (3) reported the production of antisheep hemolysins in rabbits by the injection of heart, liver, and muscle of mice. While the publication did not contain any reference to the strains used in this work, the author kindly made available to us unpublished data[1] which showed that the antibody production and the results of absorption appeared in no way dependent on the strain which was utilized (C3H, A, C alb, C57 brown, C57 black, dba, NB, Y, and C58). Antisheep agglutinins were not included in the investigation. Red cells of mice were found incapable of producing antisheep hemolysins. Brown concluded from his absorption experiments and other findings that the antigen in mouse tissues responsible for the production of antisheep hemolysins was not identical with the Forssman antigen.

The following antigens were used in our experiments: spleen, kidney, tumor tissue, liver, and erythrocytes. These tissues were derived from strains C3H, dba, and C57 black. The tumors used were: spontaneous mammary carcinoma of C3H, transplanted mammary carcinoma of dba, and methylcholanthrene-induced sarcoma of C57 black. As a rule, two rabbits were injected with each antigen. In the experiments with erythrocytes, liver, and kidney, suspensions of pooled tissues (or erythrocytes) of the respective strains were used, without regard to the titer of natural antisheep agglutinins in the animals from which the antigen was derived. In the experiments with spleen and tumor tissue, the antigens were derived from animals whose blood had been previously tested for antisheep agglutinins. For preparation of tissue suspensions from strains C3H and dba, animals which showed no natural antisheep agglutinins in the blood were selected, while tissues for C57 black antigens were derived from animals with titers of antisheep agglutinins of at least 1:8. In the absorption experiments, to be mentioned later, only tissues from animals tested for antibodies and selected in the manner just indicated were used.

The red blood cells were injected as 33 per cent suspensions, by volume, and all other antigens as 3.5 per cent suspensions by weight; intravenous injections were given every third or fourth day, with the dosage increasing from 0.25 cc. to 1.0 cc. One week after the sixth injection, samples of the rabbit serum were tested for antibodies. If the titers were low, a second course was given before the final bleeding. In some instances, further injections were given as "boosters" to supplement the supply of serum.

Table 2 shows the titers of antisheep agglutinins and hemolysins which were found in the rabbits after injection of spleen, kidney, and tumor tissue derived from the different strains. The results with erythrocytes and liver suspensions are not tabulated. Erythrocytes of the three strains tested did not elicit development of antisheep agglutinins or hemolysins in rabbits. The liver suspensions produced only low titers of antisheep hemolysins and no antisheep agglutinins, regardless of strain derivation.

Injections of kidney, spleen, and tumor tissue were followed by greater antibody response, with the titers increasing in the order mentioned. The titers of hemolysins were considerably higher than those of agglutinins, except in response to spleen. Furthermore, the agglutinins proved less stable and disappeared on storage earlier than the hemolysins. No relationship was apparent between the titers of the antibodies and the rapidity with which they developed, on the one hand, and the strain of the animal from which the antigen was obtained, on the other.

Absorption experiments were carried out in the following way: 1 part of the anti-mouse rabbit serums was mixed with 2 parts of 50 per cent suspensions of boiled mouse tissues, and the supernatant was removed after 1-hour incubation at room temperature. Antigens derived from each strain were used to absorb each rabbit serum; parallel experiments with suspensions of fresh sheep cells, boiled sheep cells, boiled guinea-pig kidney suspensions, and kaolin served as controls for completeness of absorption or for unspecific absorption, respectively.

The antisheep hemolysins produced in rabbits by injection of mouse kidney were removed by boiled suspensions of mouse kidneys regardless of the strain from which the immunizing and the absorbing tissues were derived; the same results were obtained after absorption with suspensions of boiled guinea-pig kidney, and fresh and boiled red cells of sheep. Kaolin suspensions did not re-

[1] Personal communication.

move appreciable amounts of hemolysins.

Similar results were obtained with an antimouse spleen serum. Mouse kidney and spleen were used for the absorption. Splenic tissue removed the antibodies completely, whereas minimal traces of antibodies were noted after absorption with kidney. The kidneys of C3H animals were somewhat less efficient in removing the antibodies than kidneys of dba or C57 black mice. However, the differences were neither distinct nor consistent enough to warrant any conclusion.

Antimouse tumor serum produced in rabbits was absorbed with tumors, kidneys, and red blood cells of the different strains. The results are summarized in Table 3. Absorption with tumor tissue resulted in complete removal of the antibodies; absorption with kidneys showed less complete removal, and red blood cell suspensions failed to remove any antibodies. Again the absorption was not found to depend on the strain origin of the absorbing tissues.

In Table 4 experiments are summarized in

TABLE 2

HETEROPHILIC ANTIGEN IN MOUSE TISSUES

Mouse strain	Rabbit no.		Antibody titer: Before immunization, with spleen	Antibody titer: After immunization, with spleen	Rabbit no.	Antibody titer: Before immunization, with kidney	Antibody titer: After immunization, with kidney	Rabbit no.	Antibody titer: Before immunization, with tumor tissue*	Antibody titer: After immunization, with tumor tissue*
C3H	16	A†	1	512	22	1	8	28	16	16
	"	IH	5	5,120	"	40	1,280	"	80	5,120
	"	CH	0	640	"	10	160	"	10	1,280
"	17	A	2	512	23	1	16			
	"	IH	5	5,120	"	40	1,280			
	"	CH	1	640	"	10	320			
dba	14	A	0	256	20	4	32	32	2	32
	"	IH	5	2,560	"	80	640	"	40	5,120
	"	CH	1	320	"	20	160	"	5	2,560
"	15	A	1	1,024	21	1	2	33	2	32
	"	IH	5	5,120	"	10	640	"	160	5,120
	"	CH	0	2,560	"	5	160	"	40	1,280
C57 black	18	A	2	512	24	1	0	30	2	32
	"	IH	20	5,120	"	40	640	"	80	5,120
	"	CH	5	320	"	20	80	"	10	1,280
" "	19	A	0	512	25	1	8	31	2	128
	"	IH	20	5,120	"	20	1,280	"	80	10,240
	"	CH	0	640	"	5	320	"	5	5,120

* C3H—Spontaneous mammary carcinoma.
dba—Transplanted mammary carcinoma.
C57 black—Methylcholanthrene-induced sarcoma.

† A—Titer of antisheep agglutinins.
IH—Titer of incipient hemolysis.
CH—Titer of complete hemolysis.

TABLE 3

SEROLOGIC ANALYSIS OF ANTI-MOUSE TUMOR SERUM

Strain of immunizing antigen	Rabbit no.		Antibody titers: Unabsorbed	Kaolin	After absorption with: G.P. kidney, fresh and boiled sheep RBC	Tumor (C3H, dba, C57 bl.)	Kidney (C3H, dba, C57 bl.)	RBC C3H, C57 bl.
C3H	28	A*	8	8	0	0	2	8
	"	IH	1,280	640	0	0	10	2,560
	"	CH	160	160	0	0	0	160
dba	32	A	16	16	0	0	4	32
	"	IH	5,120	1,280	0	0	40	5,120
	"	CH	1,280	320	0	0	10	1,280
"	33	A	32	32	0	0	2	
	"	IH	1,280	1,280	0	0	40	
	"	CH	320	320	0	0	0	
C57 black	30	A	16	16	0	0	4	16
	"	IH	5,120	1,280	0	0	40	2,560
	"	CH	1,280	320	0	0	0	640
" "	31	A	32	32	0	0	8	
	"	IH	10,240	2,560	0	0	160	
	"	CH	2,560	1,280	0	0	10	

* A—Titer of antisheep agglutinins.
IH—Titer of incipient hemolysis.
CH—Titer of complete hemolysis.

which two immune serums were used: (*a*) that of guinea pigs injected with red cells of sheep; (*b*) that of rabbits injected with boiled suspension of guinea pig kidney. The first serum is a true isophilic serum (serum which lacks Forssman-type antibodies because of the presence of Forssman antigen in the tissues of the guinea pig). The antibodies for sheep cells present in the second serum, on the other hand, are true heterophilic antibodies, because the tissues of the rabbit lack Forssman antigen and the antigen used in immunization is the prototype Forssman antigen. Only fresh red cells of sheep absorbed the antibodies from the isophilic immune serum. On the other hand, all the known carriers of the Forssman antigen and the kidney and tumor suspensions of mice removed the antibodies from the heterophilic immune serum, completely or in large amounts. Rabbit kidney, known to be free of Forssman antigen, lacked absorbing ability, as did the nonspecific control suspension of kaolin. Mouse kidney was a slightly less potent absorbing agent than was mouse tumor tissue; this finding is in good agreement with their respective immunizing properties. There was no difference in the behavior of kidney or tumor tissue depending on strain derivation.

TABLE 4

ANTIGENIC ANALYSIS OF MOUSE TISSUES

	Antibodies					
	Isophilic*			Heterophilic†		
	A	IH	CH	A	IH	CH
Unabsorbed	320	2560	320	40	2560	640
After absorption with:						
Fresh sheep RBC	0	0	0	0	0	0
Boiled sheep RBC	160	1,280	160	0	0	0
Guinea-pig kidney	320	1,280	160	0	0	0
Rabbit kidney	160	2,560	320	20	2,560	320
Kaolin	320	1,280	320	20	1,280	320
Mouse kidney, C3H	160	640	160	20	160	40
Mouse kidney, dba	160	640	160	0	160	40
Mouse kidney, C57 bl.	160	640	160	0	80	0
Mouse tumor, C3H	160	640	160	0	40	0
Mouse tumor, dba	160	640	160	40	80	0
Mouse tumor, C57 bl.	160	640	160	0	0	0

* Antisheep RBC guinea-pig serum.

† Anti-guinea pig kidney rabbit serum.

Antisheep agglutinins in additional inbred strains.—Since our last report (6), the total number of strains examined and of animals within the strains has increased. Table 5 presents a summary of the results now available. The largest increase in animals tested has been in strain C57 black. The percentage of zero titers in this group has increased from 3.5 to 9.8, while the percentage of titers of 16 and higher has decreased from 55.4 to 43.9. As an explanation it may be suggested that a study of genetic factors which is under way showed that there are some "sublines" of C57 black mice with considerably higher incidence of zero titers and with few high titers.

In addition to the six strains previously tested, two more have been examined, C57 brown, subline cd, and strain I.[2] Both strains showed a low incidence of natural antisheep agglutinins.

Age and antisheep agglutinins.—In animals tested in previous work (5, 6), no relationship between presence or titer of antisheep agglutinins and age was observed. The age of these animals ranged from 10 weeks to 20 months, with but few of them younger than 3 months. Subsequent work showed that animals younger than 12 weeks, regardless of strain, possessed antisheep agglutinins less frequently than older animals. For this reason, a systematic study of the relation between age and antisheep agglutinins was undertaken. Eight age groups ranging from 5 to 12 weeks, of C57 black mice, born in this laboratory,[3] were tested. Each group consisted of 30 animals, with both sexes approximately evenly distributed. More than 80 per cent (25 of 30) of the animals in the youngest age group (5 weeks) had no antibodies, even in undiluted serum. The highest titer (1:8) was found in only one animal. The incidence of antibodies

TABLE 5

ANTISHEEP AGGLUTININS IN INBRED STRAINS OF MICE

Strain	No. of animals tested	Agglutinins absent		Agglutinins present		Titers of 16 and higher	
		(No.)	(Per cent)	(No.)	(Per cent)	(No.)	(Per cent)
C57 black	326	32	9.8	294	90.2	143	43.9
C3H	122	84	68.9	38	31.1	0	0
dba	186	84	45.2	102	54.8	9	4.8
Marsh-albino	56	21	37.5	35	62.5	1	1.8
B alb C	60	21	35.0	39	65.0	0	0
Akm	87	61	70.1	26	29.9	0	0
C57 brown	91	53	58.2	38	41.8	1	1.1
I	81	67	82.7	14	17.3	0	0

[2] Strain I animals were kindly provided by Dr. John J. Bittner, Division of Cancer Biology, University of Minnesota. Strain C57 brown animals were purchased from Jackson Memorial Laboratory, Bar Harbor, Maine.

[3] The colony was bred from stock received in 1946 from Dr. A. Tannenbaum, Michael Reese Hospital, Chicago, Illinois.

was similar in the animals 6 and 7 weeks old, but changed significantly in the eighth week, when the number of animals without antibodies dropped to 15 (50 per cent); titers of 1:8 or higher were found in five animals, and 1:32 was the highest titer observed. This trend continued steadily in higher age groups, and it reached the minimum of 33 per cent of animals without antibodies and the maximum titer of 1:128 (in one animal) in the age group of 12 weeks. The comparatively small number of animals comprising each of the age groups makes it understandable that fluctuations in the antibody distribution were observed between 5 and 12 weeks, but it is apparent that around the age of 8–10 weeks there is a definite increase in the incidence of agglutinins which continues during the next weeks. It is also noteworthy that higher titers of the antibodies—1:32 and more—were found first in mice 8 weeks old and that the number of animals with these higher agglutinin levels increased during the subsequent weeks.

DISCUSSION

The experiments failed to detect antigenic differences which could account for the differences in the incidence of natural antisheep agglutinins in the strains tested. We realize that these negative findings cannot be considered to prove conclusively the absence of such antigenic differences. It is conceivable that by using whole tissues as antigens, as was done by us, differences could be masked which might become apparent if lipid fractions were to be used instead, since it is known that antigens of the Forssman type are fat-soluble. This problem is being investigated.

These results confirm previous reports on the presence of Forssman antigen in tissues of mice. Some of our results are not in agreement with those recently reported by Brown (3). The discrepancies will be discussed in a separate presentation.

The results obtained with tumor tissues, both in the immunization and the absorption experiments, showed them to possess relatively large quantities of Forssman antigen. This is in good agreement with the old observation of Morgenroth and Bieling (17, 18) on heterophilic antigens in mouse carcinoma. This work was extended to other tumors more recently by Dmochowski (7–9). The three mouse tumors used by us did not differ from each other in their content of heterophilic antigen.

In our former communications it was pointed out that the C57 black strain, which is characterized by the high incidence of natural antisheep agglutinins, shows a low incidence of spontaneous mammary carcinoma (1 per cent or less) and of other, primarily nonepithelial tumors—10–20 per cent (13). It is not possible to correlate the incidence of antisheep agglutinins with the incidence of spontaneous mammary carcinoma, since strains Akm and B alb C, with low incidence of this tumor, were also found to have a low incidence of antisheep agglutinins. Strain Akm shows a high spontaneous incidence of leukemia, and in strain B alb C internal tumors are common (13). In the C57 brown strain, subline cd, the females are reported to show a medium incidence of spontaneous mammary carcinoma (13). The literature on strain I was reviewed recently by Andervont (1). Spontaneous malignant tumors are rare among these animals; the gastric lesions, which occur in almost all animals, are considered benign, in spite of their peculiar behavior in transplantation experiments (2). Hence, it is apparent that no relationship can be shown to exist between the occurrence of natural antisheep agglutinins and the tendency to development of spontaneous tumors in the individual strains.

The absence of a relationship between the natural antisheep agglutinins and the presence of the milk agent was reported.[4]

The observations in young mice suggest that testing for antisheep agglutinins should not be done in animals less than 12 weeks old if one wants to establish the incidence of the antibodies in a particular strain. With the exception of the few animals tested some time ago, we have followed this rule.

The findings are in accord with previously recorded observations that natural hemoantibodies develop postnatally, a phenomenon which was designated by Hirszfeld (14) as "serologic maturation." This phenomenon is well known from studies on human isoagglutinins (4, 20, 21). Similar observations on heteroagglutinins in chicks have been recently reviewed and confirmed (15).

The gradual postnatal development of antisheep agglutinins does not offer any clue as to why they are found so much more frequently in C57 black mice, as compared with mice of other strains. It has been shown that heterophilic antibodies may owe their formation to antigens introduced in the food (10, 19). It may be that natural antibodies have a similar origin. Such an assumption could have hardly any bearing on the present problem, since C57 black animals and animals of the other strains received the same stock diet (Rockland mouse diet). It may also be that natural

[4] Davidsohn, I., Stern, K., and Bittner, J. J. Milk Agent and Natural Antisheep Agglutinins in Mice of Inbred Strains. Proc. Soc. Exper. Biol. & Med. (in press).

agglutinins develop in response to enteral immunization by intestinal saprophytes. Differences in the composition of the intestinal flora of various strains have not to our knowledge been studied.

SUMMARY

1. Natural antisheep agglutinins have been studied thus far in strains C57 black, C3H, dba, Marsh-albino, B alb C, Akm, C57 brown, and I.

2. The antisheep agglutinin in mice is not of the Forssman type.

3. Antigen of Forssman type was found in normal and neoplastic tissues of C3H, dba, and C57 black mice, independent of strain. The antigen was not present in the erythrocytes.

4. No relationship was noted between presence and titer of natural antisheep agglutinins and the incidence of spontaneous tumors in the strains studied.

5. The incidence and titer of antisheep agglutinin in C57 black mice showed a progressive rise from the fifth to the twelfth weeks of life.

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Failure To Inhibit the Formation of Mammary Carcinoma in Mice by Intermittent Fasting*

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Chronic restriction of caloric intake inhibits the formation of various types of tumors in the mouse. This inhibition is evidenced both by a decrease in the proportion of mice developing tumors and by a delay in the time at which the tumors appear (4–6, 8, 11, 15, 16). The magnitude of the inhibitory effect is dependent on the degree of caloric restriction (1, 10, 14), the type of tumor (11), the dosage or potency of the carcinogen (12), and on whether the restricted caloric intake is maintained during the stage of initiation or the stage of development of the tumor (9). In all these studies the daily dietary restriction of caloric intake was continued for months—in some instances for almost the whole life span of the mice. The question arose as to whether continued daily caloric restriction is necessary to inhibit the formation of tumors or whether comparable effects might be observed in animals subjected to intermittent fasting. The following experiment was planned to determine the effect of fasting for 24-hour periods twice weekly, with no other limitation of food intake.

EXPERIMENTAL

One hundred and four female mice of the dba inbred strain, raised in our laboratory, were divided into two equal groups by random selection. The mice were 34 weeks of age, and each had delivered and raised one litter at about 3 months of age. They had been fed Purina laboratory chow since weaning. For the study they were housed in sets of five, in cages with solid bottoms.

The diet employed was composed of Purina Fox Chow meal, 50 per cent; skimmed milk powder, 25 per cent; and cornstarch, 25 per cent. Drinking water was available at all times. The mice of the control group, A 101, were fed *ad libitum* daily. Those of the experimental group, A 102, were given the same diet, except that the food was withheld from them every Monday and Thursday (in addition, the cages were cleaned at about 9:00 A.M. on those mornings); on Tuesday and Friday mornings the mice of this group were given a double weight of the ration. Although the mice of group A 102 fasted 2 days during each week, their food intake was *ad libitum* on a weekly basis.

Each mouse was numbered and its course recorded separately. At biweekly intervals the animals were weighed and inspected for general appearance and neoplasms. All mice underwent autopsy at death or at the termination of the experiment. Tumors were recognized grossly; for confirmation, microscopic sections were made of a considerable proportion of the mammary carcinomas, as well as of the few metastases and other lesions found.

The experiment proceeded smoothly and was terminated when the few remaining mice were 113 weeks old.

The average daily food consumption during the study was 3.2 gm. per mouse for group A 101 and 3.1 gm. for group A 102. The over-all effect of the periodic fasting regimen on the mean body weight of the mice was a slight depression, as shown in Table 1.

TABLE 1

EFFECT OF INTERMITTENT FASTING ON BODY WEIGHT OF MICE

GROUP*	AGE OF MICE, WEEKS					
	34†	40	50	60	70	80
	Mean body weight, gm.					
A 101	27	27	29	31	31	30
A 102	27	27	28	30	29	28

* A 101, fed *ad libitum* daily; A 102 fasted twice each week for 24 hr.
† Experiment began when mice were 34 weeks old.

At various times during the experiment the mice of group A 102 were weighed just preceding the fasting period, at the end of the 24-hour fasting period, and again 24 hours after refeeding. The 24-hour fast resulted in an average weight loss of

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3 gm. (approximately 10 per cent of body weight). Within the first 24 hours of realimentation the mice approximated their pre-fasting body weight.

The effect on the mice of fasting twice weekly for 24 hours is shown in the cumulative tumor curves (Fig. 1); other comparative factors of tumor formation are given in Table 2.

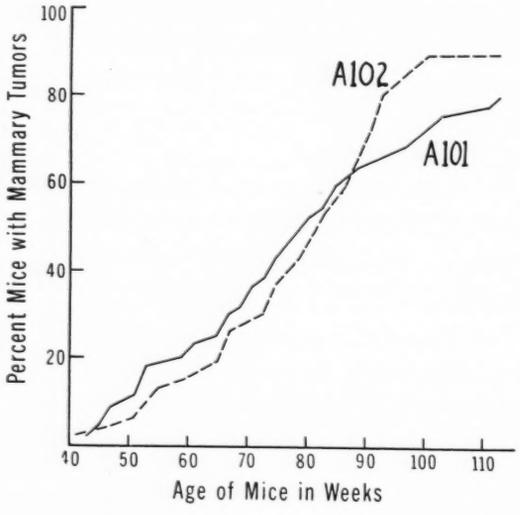

FIG. 1.—Cumulative curves showing formation of spontaneous mammary carcinoma. Group A 101 fed *ad libitum* daily; group A 102 fasted twice weekly for 24 hours and fed *ad libitum* between fasting periods.

TABLE 2

EFFECT OF INTERMITTENT FASTING ON INCIDENCE AND TIME OF APPEARANCE OF MAMMARY CARCINOMA IN DBA MICE

GROUP*	NUMBER† OF MICE	MICE FORMING MAMMARY CARCINOMA		AGE AT TUMOR APPEARANCE, WEEKS		NUMBER OF MICE TUMOR-FREE AND ALIVE AT 113 WEEKS‡
		(Number)	(Per cent)	(Range)	(Mean)	
A 101	44	35	80	43–113	74.5±3.2	2
A 102	46	41	89	41–101	77.7±2.4	2

* A 101, fed *ad libitum* daily; A 102 fasted twice each week for 24 hr.

† There were 52 mice in each group initially; the number of mice indicated represents a calculated adjustment to correct for the deaths of nontumor mice during the experiment (2).

‡ End of experiment.

Both indices of tumor formation—per cent of mice forming mammary carcinoma and the mean time of appearance of the tumors—indicate that periodic fasting, under the experimental conditions outlined, had no inhibitory effect upon the formation of spontaneous mammary tumors. There was also no effect upon the proportion of tumor-bearing mice that developed multiple mammary tumors—31 per cent and 29 per cent for groups A 101 and A 102, respectively.

The mice that developed tumors were continued on their particular dietary regimen, and the tumors were measured at 1-week intervals to determine their rate of growth. The periodic fasting regimen (imposed on the mice of group A 102) had no effect on the rate of growth of the tumors nor on the time of survival of the animals after tumor appearance (Table 3).

TABLE 3

EFFECT OF INTERMITTENT FASTING ON GROWTH OF MAMMARY CARCINOMA AND SURVIVAL OF TUMOR-BEARING MICE

GROUP*	GROWTH INDICES OF MAMMARY CARCINOMA†		SURVIVAL TIME, WEEKS‡	
	(Range)	(Mean)	(Range)	(Mean)
A 101	1.4–13.5	6.2±0.49	4–17	9.1±0.62
A 102	1.2–15.4	5.8±0.55	0–17	8.5±0.66

* A 101 fed *ad libitum* daily; A 102 fasted twice each week for 24 hr.

† Mean daily increment of sum of major and minor axes of tumor in units of 0.1 mm.; 32 tumor-bearing mice measured in A101; 35 in A 102.

‡ Interval between detection of the tumor and death of the animal.

DISCUSSION

Mice that fasted for 24 hours twice weekly (Monday and Thursday)—eating *ad libitum* on the other days of the week—developed spontaneous mammary tumors in about the same percentage as mice fed *ad libitum* daily. This was not unexpected, inasmuch as the dietary regimen resulted in only a slight decrease in the over-all caloric intake and a slight retardation in the weight of the mice (13).

Actually, the group that fasted intermittently developed a slightly higher percentage of tumors than the controls, and this occurred through an increased rate of formation in the mice of group A 102 after they were 70 weeks old (Fig. 1). It is not concluded from this single experiment that this augmentation is real. Yet, the augmentation might have occurred through the stress on the tissues, particularly the liver, or because the animal's caloric requirement during the fasting period was obtained through the use of fat from its depots—in this period the mice might be considered to be on a high fat diet (7). Further experimentation, starting with younger mice, would be needed to clarify this possibility.

A study related to the present experiment is reported by Carlson and Hoelzel (3), who investigated the effects of intermittent fasting on the prolongation of life in rats. They concluded from incidental findings with relatively few animals that "the development of mammary tumors was retarded in proportion to the amount of fasting." However, while there appears to have been an effect in the rats that fasted 1 day in 2, tumor formation in those that fasted 1 day in 3 or 4 did not differ significantly from that in the controls.

On the basis of the present experiment and previous reports, it is our opinion that, while chronic caloric restriction strikingly inhibits tumor formation, intermittent or periodic caloric restriction does not have this effect unless the dietary regimen is such that the body weight of the animals is significantly less than that of the *ad libitum* controls.

SUMMARY

Intermittent caloric restriction—mice fasting twice weekly for 24 hours, followed by *ad libitum* feeding between fasting—did not materially affect the mean food consumption or mean body weight of dba female mice. There was no inhibitory effect on the incidence or rate of formation of spontaneous mammary carcinoma or on their subsequent growth.

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Vaginal Sensitivity to Estrogen as Related to Mammary Tumor Incidence in Mice

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The demonstration of the importance of ovarian secretion or exogenous estrogen to a high mammary tumor incidence in mice was followed by numerous studies of the estrous cycles of mice of different strains, in an attempt to correlate high mammary tumor incidence with some peculiarity of the cycle. In general, no significant and consistent correlation could be demonstrated in this regard. Korteweg and co-workers, however, focused attention on the possibility that, whereas outward manifestations of the cycle might be relatively constant, fundamental differences may exist in the sensitivity of the genital tissues to estrogen, and that such differences may be related to the mammary tumor incidence. Van Gulik and Korteweg (5) reported that of one high tumor and two low tumor inbred strains, compared with regard to vaginal estrogen-sensitivity, the high tumor dba strain was the most resistant to exogenous estrogen stimulus, requiring roughly 3 times the amount of estrogen needed by the low tumor C57 strain for a comparable degree of vaginal stimulation. They pointed out also that Bonser (1) observed a somewhat higher percentage of positive vaginal smears in a low mammary tumor strain than in a high mammary tumor strain when both were injected with equal but varying doses of estrogen. On the basis of such findings, they theorized that, since a normal estrous cycle is essential for continued existence, those strains of low vaginal estrogen-sensitivity probably produce larger amounts of estrogen and that this may be causally related to a high mammary tumor incidence. They reported also that reciprocal hybrids between the dba and C57 strains showed an almost equal vaginal sensitivity to estrogen, intermediate between that of the parental strains, indicating that vaginal susceptibility to estrogen bears no relation to the maternal extrachromosomal factor.

Mühlbock (2, 3), from the same laboratory, confirmed the higher estrogen requirement (5–7 times as much) of the high tumor dba strain as compared to the low tumor C57 and 020 strains for a comparable degree of vaginal stimulation by either intravaginal or subcutaneous administration of estrogen.

Shimkin and Andervont (4) also reported on the vaginal estrogen-sensitivity of three strains of mice of known mammary tumor incidence—the C57, C, and C3H strains. Again the high tumor strain was more resistant, the C3H strain requiring twice as much estrogen as the low tumor C57 and C strains for a positive response in 50 per cent of the mice. It was also reported that, whereas foster nursing of the high tumor strain by a low tumor strain, or vice versa, altered the mammary tumor incidence, it did not alter vaginal sensitivity to estrogen.

The present investigation was undertaken to determine whether the reported inverse relationship of vaginal estrogen-sensitivity and mammary tumor incidence is of universal occurrence or restricted to the few inbred strains of mice studied thus far. In view of the apparent lack of effect of milk factor on vaginal estrogen-sensitivity, it would seem improbable that such an inverse relationship could apply to all strains of mice, since some strains may have a low mammary tumor incidence only because of the absence of the milk factor. Nevertheless, such an inverse relationship might conceivably apply when comparison is made only between strains known to possess the milk factor.

MATERIALS AND METHODS

Included in the present study are mice of three strains of high mammary tumor incidence, the C3H, CBA, and A strains, and three of low tumor incidence, the C57, JK, and N strains. In addition, mice of two high tumor strains free of milk factor

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as a result of foster nursing are included. These are the C3H and A strains, designated, respectively, BL and AL. Also studied were three groups of F1 hybrids, the CC1 (C57 ♀ × CBA ♂), the CC2 (CBA ♀ × C57 ♂), and the AB2 (CBA ♀ × A♂).[1]

The mammary tumor incidence of breeding females of the inbred strains is presented in Table 1. These data are compiled largely from Dr. Gardner's colony over the period of approximately the past ten years and from the relatively fewer animals of the author's colony during the past two years. Both colonies were maintained under comparable conditions. Breeding mice received Purina laboratory chow, and nonbreeding experimental animals received Purina Fox Chow. Adequate numbers of animals are not available to report mammary tumor incidence for the N strain under comparable conditions. However, mammary tumors are rare in mice of this strain in Dr. Strong's colony, and none have been observed in the few breeding females used for the present experiment.

TABLE 1

INCIDENCE OF MAMMARY ADENOCARCINOMA IN BREEDING FEMALE MICE LIVING 150 DAYS OR MORE

Strain	No. of mice	Per cent with mammary tumors	Strain	No. of mice	Per cent with mammary tumors
C3H	369	83	JK	37	0
CBA	154	67	AL	50	0
A	156	58	BL	258	6.2*
C57	138	0			

* In addition to the above-mentioned adenocarcinomas in the BL strain, seventeen mice, or 6.7 per cent, had at autopsy one or more small nodular foci of squamous metaplasia of the mammary glands. Four more animals, or 1.6 per cent, had squamous tumors of 4–15 mm. diameter. The two smaller tumors were adenomas containing squamous cysts. The largest was a hard, white, granular, rapidly growing squamous-cell carcinoma with scattered small areas of adenocarcinoma. The fourth was a 1-cm. hard, white, granular tumor mass, on which sections are not available.

Groups of approximately twenty virgin female mice of each of the stocks mentioned previously were ovariectomized at approximately 1 year of age (8–14 months range), allowed to rest for 5–7 weeks, then smeared 4 times on alternate days. In the JK strain many of these smears contained, in addition to leukocytes, numerous small nucleated epithelial cells, indicating a possible extra-ovarian source of estrogen. After estrogen administration, the smears became fully cornified, followed by a completely diestrous type of smear. It is probable, therefore, that during the course of the assay, involving repeated estrogen injections, the amount of endogenous estrogen was negligible. Nevertheless, the JK strain is included in the present report with the reservation that it may possess a post-castration extra-ovarian source of estrogen, in the absence of which responsiveness to exogenous estrogen might have been lower.

A single injection of estradiol benzoate[2] in 0.05 cc. of sesame oil was used. Smears were taken by lavage, and only a fully cornified smear with no more than occasional leukocytes was considered positive. The mice were primed with 0.1 μg of estradiol benzoate, and assays were performed at 1–2-week intervals thereafter, depending on the amount of the previous injection and the length of time required for a return to a negative smear. A graded series of estrogen dosages were administered, and different dose levels repeated as often as necessary to obtain a fairly smooth sigmoid type dose-response curve for each strain.

The dams of the mice used for vaginal assay were kept to determine mammary tumor incidence. Two of the BL dams developed mammary tumors—one an adenocarcinoma and the other a squamous-cell carcinoma with small scattered areas of adenocarcinoma. Progeny of both of these mice were eliminated from the experimental series.

The incidence of mammary adenocarcinomas in the BL strain, since it was obtained from Dr. Bittner in 1941, has been 6.2 per cent. These tumors have appeared relatively more frequently in recent years, raising some question as to the complete absence of milk factor in this strain at the present time. The data for the BL strain are nevertheless presented, since the tumor incidence is still much below that of the C3H strain, and progeny of tumor-bearing animals have been eliminated.

RESULTS AND DISCUSSION

The percentage of positive vaginal smears obtained from mice of the different strains at the various levels of estrogen are presented in Table 2. The dose-response curves for the different strains are presented graphically in Figures 1, 2, 3, and 4. Table 3 lists the different strains in decreasing order of vaginal sensitivity, as measured by the minimum dose of estrogen required for a positive response in approximately 50 per cent of the mice.

The dose-response curves of the three high tumor and the three low tumor inbred strains do not affirm the reported inverse relationship of vaginal estrogen-sensitivity and mammary tumor incidence. The range of sensitivity represented by the two groups of curves overlaps considerably. It is apparent why an inverse relationship might be indicated in the comparison of a smaller number of strains, one of which is the low tumor C57

[1] The BL and AL strains were obtained from Dr. W. U. Gardner who, in turn, received them from Dr. J. J. Bittner in 1941 and 1944, respectively. The remaining inbred strains were obtained from Dr. Gardner and Dr. L. C. Strong.

[2] The estradiol benzoate was generously provided by the Schering Corporation.

strain. This proved to be the most sensitive of the several strains tested.

If one considers only those inbred strains known to possess milk factor, the C3H, CBA, and A strains, the mammary tumor incidence of these strains is directly proportional, rather than inversely proportional, to vaginal estrogen-sensitivity. Even this relationship does not apply if one also considers the fact that the C57 strain, after exposure to the milk factor, generally shows a relatively low incidence of mammary tumors, while vaginal estrogen-sensitivity remains unaltered

TABLE 2

PERCENTAGE OF POSITIVE VAGINAL SMEARS OBTAINED FROM MICE OF DIFFERENT STRAINS TREATED WITH GRADED DOSES OF ESTROGEN

MICROGRAMS OF ESTRADIOL BENZOATE	STRAIN OR HYBRID GROUP										
	C57	JK	C3H	BL	CC1	CC2	CBA	AB2	N	A	AL
	Per cent positive/Number of smears										
0.4		95/22			95/22	91/23	95/21	90/21	94/17	89/19	100/18
0.3	100/23	82/22	100/24	100/11	86/22	100/23	87/39	81/21	82/17	89/19	79/19
0.27									56/32	55/38	58/36
0.25								76/21	44/16	42/19	33/18
0.22								76/21			
0.20		70/44	79/68	81/32	86/44	76/45	72/58	52/42	29/17	16/19	6/18
0.15		61/44	76/21	60/10	77/44	71/45	51/41	29/21			
0.12							38/21				
0.10	96/23	59/22	52/46	61/33	64/44	53/47	24/21	19/21	29/17	16/19	5/19
0.08		50/22	8/24	0/11	23/44	22/46					
0.06	46/46	23/22									
0.05	9/23										
0.04	11/46	0/22									

TABLE 3

MINIMUM AMOUNT OF ESTROGEN GIVING POSITIVE VAGINAL SMEARS IN APPROXIMATELY 50 PER CENT OF THE MICE

Strain	Micrograms of estradiol benzoate	Strain	Micrograms of estradiol benzoate
C57*	0.06	CBA†	0.15
JK*	0.08	AB2	0.2
C3H†	0.1	N*	0.25–0.27
BL	0.08–0.1	A†	0.25–0.27
CC1	0.08–0.1	AL	0 27
CC2	0.1		

* Low mammary tumor inbred strains.
† High mammary tumor inbred strains.

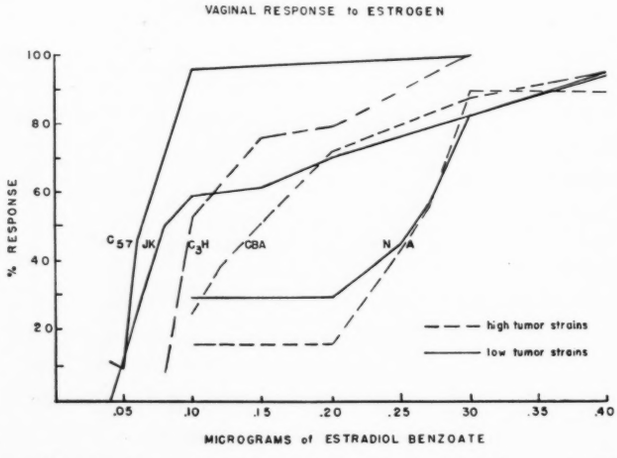

FIG. 1.—Percentage of positive vaginal smears from mice of three high tumor and three low tumor strains treated with various levels of estrogen.

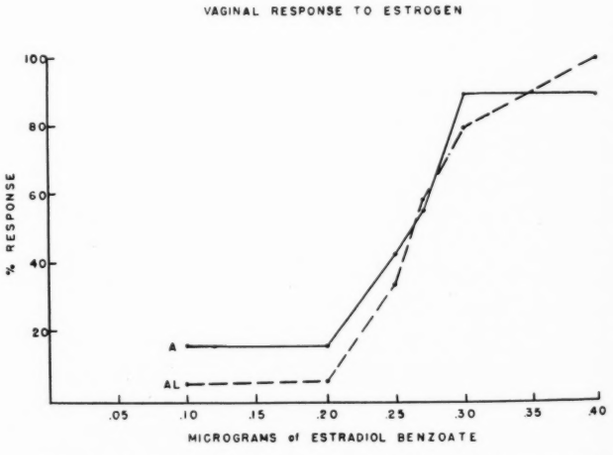

FIG. 2.—Percentage of positive vaginal smears from mice of the A strain and fostered A strain treated with various levels of estrogen.

(4). In short, no consistent correlation is demonstrable between mammary tumor incidence and vaginal sensitivity to estrogen.

Mice of the A and AL strains, and of the C3H and BL strains, showed remarkably similar vaginal response to the different levels of estrogen. The presence or absence of milk factor as a result of foster nursing did not significantly alter vaginal sensitivity to estrogen. This is in agreement with the findings of Shimkin and Andervont (4). The similarity of these two sets of dose-response curves is also of interest in view of the many years during which the lines of descent of the fostered and non-

fostered mice of these two stocks have been separated.

The dose-response curves for the three groups of hybrids used and for the three parental inbred strains from which they were derived are presented in Figure 4. The reciprocal hybrids between the high tumor CBA and the low tumor C57 strains show a vaginal sensitivity intermediate between that of the two parental stocks; whether the female parent was from the high tumor or low tumor strain made very little difference. As mentioned above, similar results have been reported by Van Gulik and Korteweg (5), using reciprocal hybrids between the dba and C57 strains.

The AB2 hybrids between the two high tumor strains, CBA and A, also show a vaginal estrogen-sensitivity intermediate between that of the parental strains.

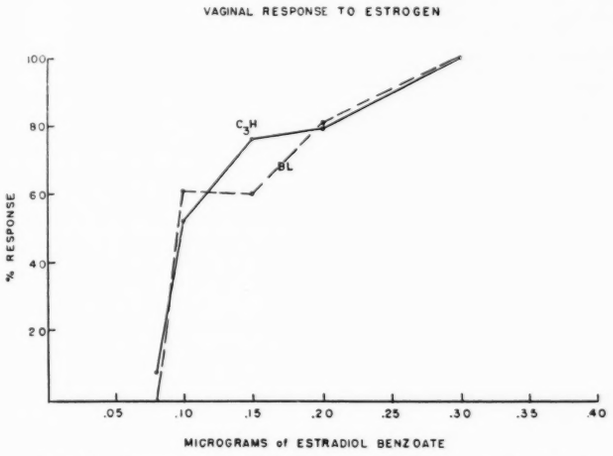

FIG. 3.—Percentage of positive vaginal smears from mice of the C3H and fostered C3H strains treated with various levels of estrogen.

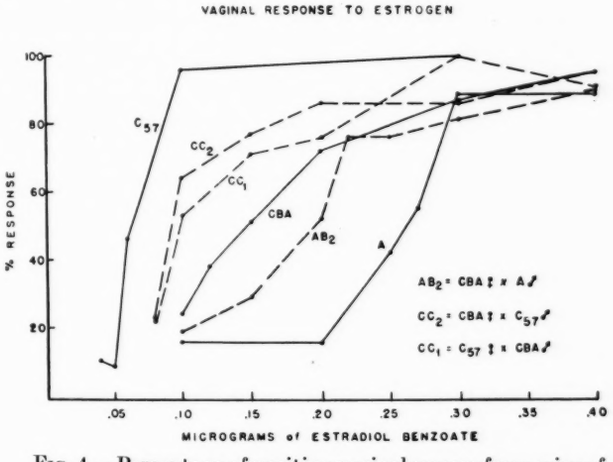

FIG. 4.—Percentage of positive vaginal smears from mice of three hybrid groups and the three parental inbred strains treated with various levels of estrogen.

SUMMARY

1. The inverse relationship between vaginal sensitivity to estrogen and mammary tumor incidence that has been reported for limited numbers of strains of mice was found not to apply when larger numbers of strains were studied. This was the case when either all strains or only those known to possess milk factor were considered.

2. The presence or absence of milk factor, obtained by foster-nursing or by reciprocal crossing of a high and a low tumor strain, did not alter vaginal sensitivity to estrogen.

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Book Reviews

Cell Growth and Cell Function. A Cytochemical Study. By T. O. Caspersson. New York: W. W. Norton & Co., 1950. Pp. 185. $5.00.

This book is a report on cytochemical research carried out by Caspersson and his co-workers from 1932 to 1948. It is based on the author's Thomas W. Salmon Memorial lectures.

The first half of the book contains a description of Caspersson's well-known ultraviolet microspectrophotometric method; it refers to the difficulties involved in the development of the method and to the way they were solved, and it points out the conditions under which the method is applicable, as well as its accuracy and limitations. The treatments to be applied to the biological objects under investigation are briefly described. As an illustration, a few examples of the application of the method are given; they include the localization and determination of nucleic acids and of proteins rich in tryptophan, tryosine, and diamino acids in different regions and organelles of the cell.

The second half of the book deals with the changes in distribution and concentration of nucleic acids and proteins rich in diamino acids which occur during the mitotic cycle, normal and abnormal growth, protein secretion, excitation of nerve cells, and multiplication of bacteria, yeasts, and animal viruses.

Since this report is presented in the form of lectures, only the main results and general conclusions are given, together with absorption spectra and very good photographs; the detailed experimental results are to be found in the author's original papers, to which abundant reference is made.

It is clearly stated in the introduction that the presentation is confined to the results obtained by the Stockholm group. We believe that the book would have gained much if at least some reference had been made to research carried out by other laboratories in the same field or in closely related fields, e.g., to the numerous cytochemical data obtained by other methods, to biochemical and chemical studies on isolated nuclei and cytoplasmic particles, to other investigations of protein synthesis, virus multiplication, and to data from genetics. This would have given a key to the literature to readers not specially acquainted with this field, to whom the book appears to be addressed.

As for the theories of protein and virus duplication presented in very suggestive schemes, we are of the opinion that they could lead to interesting working hypotheses accessible to experimental checking provided they are not taken for granted, but rather considered together with experimental results obtained in other fields.

The book is attractive, abundantly illustrated with good photographs and diagrams (94 figures for 185 pages); it is well printed and easy to read. It will prove valuable in again calling the attention of investigators to the striking changes occurring in the distribution and concentration of nucleic acids and some protein components during protein synthesis, nerve activity, and normal or abnormal growth.

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Electron Microscopy. By Ralph W. G. Wyckoff. New York: Interscience Publishers, Inc., 1949. Pp. 248. $5.00.

This monograph on electron microscopy will be of interest to the chemist, metallurgist, physicist, and biologist. A consideration of the similarities and differences with respect to optical microscopy will be helpful for the beginner. The chapters dealing with particulate suspensions, thin sections, membranes and surface films, replicas and metal shadowing will be useful to the expert. A survey of the capabilities and the inadequacies of the commercial models which are available will aid in deciding what type of instrument one needs for the investigation at hand. A review of the more common idiosyncrasies of specific instruments, as well as illustrations of differences in engineering of several commercial types, should prove helpful in attaining peak performance with the model one is using. Chapters devoted to the different methods by which specimens may be prepared before suitable examination is possible indicate where improvements are desirable. The difficulties which are inherent in the preparation of material and the means employed by the author in avoiding such trouble are considered. The subject matter in the monograph is presented in ten short chapters, each of which contains a bibliography. There are over 200 photographs in the second half of the book which show with what success the author has been able to photograph the test material in his investigations. There are photographs of surfaces of metal, bone and teeth, plant and animal viruses, bacteria, cellulose, nylon, collagen, fibrin, and macromolecules. The monograph adequately illustrates some of the objects which may be examined with this instrument. It also calls attention, wherever indicated, to the potential future improvements in the construction of the electron microscope and the treatment of material for examination.

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Announcements

GRANTS AND FELLOWSHIPS IN CANCER RESEARCH

The Committee on Growth of the National Research Council, acting for the American Cancer Society, is accepting applications for grants and fellowships. Applications for new grants will be received until October 1. Investigators now receiving grants will be notified individually regarding application for the extension of these grants. Final decision on grant applications will be made early in 1951. Grants approved at this time ordinarily will become effective July 1, 1951.

Fellowships available include both Fellowships in Cancer Research, of the American Cancer Society, and Damon Runyon Clinical Research Fellowships, financed by a grant from the Damon Runyon Memorial Fund to the American Cancer Society. Fellowship applications may be submitted at any time. Those received prior to November 1 will be acted upon by the Committee on Growth in December. Those received between November 1 and March 1 will be acted upon in April. Fellowships ordinarily will begin July 1, although this date may be varied at the request of the applicant.

During the past year the American Cancer Society, Inc., on recommendation of the Committee on Growth, has awarded grants and fellowships approximating $2,000,000. A program of similar magnitude is contemplated for the coming year.

Communications should be addressed to the Executive Secretary, Committee on Growth, National Research Council, 2101 Constitution Avenue, N.W., Washington 25, D.C.

GRANTS FOR ENVIRONMENTAL CANCER RESEARCH

The Committee on Growth of the National Research Council, adviser for research to the American Cancer Society, announces the formation of a Panel on Environmental Cancer with the following membership: Dr. Willard Machle, Chairman; Drs. Francis Heyroth, George H. Gehrmann, Herman Lisco, and Norton Nelson.

Increasing realization of the importance of further research in environmental cancer led to the creation of this new panel which, at the outset, will concern itself with an evaluation of the status of knowledge in this field and with the formulation of criteria for the establishment of valid relationships between environment and occupation and the occurrence of cancer. The panel also will review applications for grants in support of research in these areas. These applications, as with others submitted to the Committee on Growth, will be received until October 1, 1950.

Communications regarding grants may be addressed to the Executive Secretary, Committee on Growth, National Research Council, 2101 Constitution Avenue, N.W., Washington 25, D.C.